The Role of Pro-Resolution Mediators in Human Parturition

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ABSTRACT

Preterm labour and dysfunctional term labour lead to the most pre-eminent problems in obstetric practice today. We currently lack effective strategies for preventing spontaneous preterm labour, and prematurity remains the leading cause of perinatal morbidity and mortality. In contrast, some women do not labour effectively at term, or pregnancy becomes prolonged, both of which increase the risk of caesarean section, rates of which are on the rise in this country.

Human labour is an inflammatory process, characterised by an influx of inflammatory cells into the myometrium and cervix at the onset of labour, and an increase in production of proinflammatory cytokines and expression of cell adhesion molecules. A substantial body of evidence has now accumulated in support of this theory, but relatively little attention has yet focused on anti-inflammatory pathways within this process. The resolution of inflammation is now recognised to be an active process, driven by a variety of endogenous pro-resolution factors. These include the lipid mediator lipoxin A4 (LXA4), derived from arachidonic acid and signalling through a G-protein coupled receptor, a member of the formyl peptide receptor family, FPR2/ALX. Another is Annexin A1 (ANXA1), a glucocorticoid-regulated peptide that also signals via FPR2/ALX. The aim of this thesis was to explore the role of these anti-inflammatory and pro-resolution mediators in myometrial and placental tissues and examine what role they may have within the inflammatory process of labour.

Expression of the receptor FPR2/ALX and enzymes involved in the synthesis of LXA4 was determined by quantitative RT-PCR in labouring and non-labouring tissues of the reproductive tract and FPR2/ALX was localised in these tissues using immunohistochemistry. It was found that expression of the receptor and the enzyme 5-lipoxygenase (ALOX5) were upregulated in labouring tissues and that the receptor localised primarily to immune cells. A microarray and functional genomics were used to examine inflammation in myometrium and interesting parallels were drawn between artificially-induced inflammation and the process of labour, identifying pathways of interest for further study. Myometrial tissue was cultured in hypoxia, a

pro-inflammatory environment, and it was demonstrated by ELISA and quantitative RT-PCR that LXA4 production was unaffected in these conditions, but that there may be changes in expression of ALOX5 and FPR2/ALX.

Quantitative RT-PCR and immunohistochemistry were used to explore the expression of ANXA1 in labouring and non-labouring tissues and found localisation to immune cells and vascular endothelial cells but no difference in expression in labour. Tissue culture and quantitative RT-PCR demonstrated glucocorticoid-regulation of FPR2/ALX but not ANXA1 in myometrium.

This work has shown that LXA4 and ANXA1 are present in the reproductive tract during pregnancy and parturition and that a mechanism may exist, via upregulation of their common receptor, for a role within the inflammatory process of labour. Further work will aim to clarify the nature of this role and its functional effects, with the ultimate aim of better understanding the process of inflammation during parturition leading to the development of strategies to prevent or treat preterm and dysfunctional term labour.

DECLARATION

The thesis composition herein is my own. Where I have been a member of a research group, I have made a substantial contribution to the work, and this contribution is indicated clearly in the text. Any contribution made by others is acknowledged. This thesis has not been submitted in candidature for any other degree, postgraduate diploma or professional qualification.

Chapter 3

I acknowledge the assistance of Dr David Maldonado-Perez, who designed the primer/probe sets used in the quantitative RT-PCR experiments, performed the ELISA assay and helped prepare the immunofluoresence images.

Chapter 4

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Chapter 6

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All chapters

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Ellen Golightly, October 2011

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This thesis is dedicated to my late father, Dr Frank Golightly.

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ABBREVIATIONS

11βHSD1 and 2	11β-hydroxysteroid dehydrogenase 1 and 2
15d-PGJ(2)	15-deoxy-delta(12,14)-prostaglandin J(2)
15-epi-LXA4	15-epimer lipoxin A4
15S-HETE	15S-hydroxyleicosatetranoic acid
7-TM	Seven Transmembrane
AA	Arachidonic Acid
ABC	ATP-binding cassette
ALOX12	12-lipoxygenase
ALOX15	15-lipoxygenase
ALOX5	5-lipoxygenase
ANOVA	Analysis of Variance
ANXA1	Annexin A1
ATL	Aspirin-Triggered Lipoxin
ATP	Adenosine Triphosphate
BLT1	Leukotriene B4 receptor
BMI	Body Mass Index
BSA	Bovine Serum Albumin
c/s	Caesarean Section
CCL2	Chemokine (C-C motif) Ligand 2
cDNA	Complementary DNA
CF	Cystic Fibrosis
CMKLR1	Chemokine-Like Receptor 1
CO_2	Carbon dioxide
COX1/2	Cyclo-oxygenase 1/2
CRH	Corticotrophin Releasing Hormone
CSF3	Colony Stimulating Factor 3
Ct	Cycle Threshold
DAB	3,3-diaminobenzidine
DAPI	4,6-diamidine-2-phenylindole, dihydrochloride
DC	Dendritic Cell
DE	Differentially Expressed
DHA	Docosahexaenoic Acid
DNA	Deoxyribonucleic Acid
dNTP	deoxynucleotide triphosphates
ECM	Extracellular Matrix
EDD	Estimated Due Date
ELISA	Enzyme Linked Immunosorbance Assay
EPA	Eicosapentanoic Acid
ER	Estrogen Receptor
FACS	Fluorescence-activated Cell Sorting
FDR	False Discovery Rate
FPR1 and 3	Formyl Peptide Receptor 1 and 3
FPR2/ALX	Formyl peptide receptor 2
GC	Glucocorticoid
GCSF	Granulocyte Colony Stimulating Factor
GPCR	G Protein-Coupled Recptor

HIV1	Human Immunodeficiency Virus 1
HPA	Hypothalamic-Pituitary-Adrenal
HRP	Horseradish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM1	Intercellular Adhesion Molecule 1
IL-1, -6, -8 etc	Interleukin 1, Interleukin 6, Interleukin 8 etc
IL-1R1	Interleukin 1 Receptor 1
IL-1R2	Interleukin 1 Receptor 2
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
IUPHAR	International Union of Basic and Clinical Pharmacology
L	Labouring
LMP	Last Menstrual Period
LPS	Lipopolysaccharide
LTA4	Leukotriene A4
LTB4	Leukotriene B4
LXA4	Lipoxin A4
LXB4	Lipoxin B4
MCP-1	Monocyte Chemotactic Peptide 1
MMP	Matrix Metalloproteinase
mRNA	Messenger RNA
NF-κB	Nuclear Factor Kappa B
NGS	Normal Goat Serum
NL	Non-labouring
O_2	Oxygen
OT	Oxytocin
OTR	Oxytocin Receptor
P/S	Penicillin/Stretomycin
PARP1	Poly (ADP-ribose) polymerase-1
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PG	Prostaglandin
PGDH	Prostaglandin dehydrogenase
PGI2	Prostacyclin
PMN	Polymorphonuclear Neutrophil
PR	Progesterone Receptor
PRR	Pattern Recognition Receptors
PUFA	Polyunsaturated Fatty Acid
RNA	Ribonucleic Acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcription
RT-PCR	Real Time PCR
SAA	Serum Amyloid A
SEM	Standard Error of the Mean

SOP	Standard Operating Procedure
TGF-β	Transforming Growth Factor Beta
TIMP	Tissue Inhibitor of Metalloproteinases
TLR	Toll Like Receptor
TNF-α	Tumour Necrosis Factor alpha
TXA2	Thromboxane A2
UPL	Universal Probe Library
USS	Ultrasound Scan
VEGF	Vascular Endothelial Growth Factor

1. Literature Review

1.1. INTRODUCTION

The mechanisms that initiate and drive human parturition are not fully understood, and despite great advances in medical science over the last century, maternal and neonatal morbidity and mortality caused by dysfunctional or preterm labour remain a significant threat to women and their infants worldwide. In the UK, dysfunctional or delayed parturition at term causes significant complications including caesarean section, for which the rate is rising, having increased from 9% in 1980 in England and Wales to 21% in 2001 (Thomas 2001). Worldwide, the World Health Organisation estimate that 1000 women die every day due to complications encountered during pregnancy and childbirth. Preterm birth is the major cause of neonatal mortality and morbidity in developed countries (Norman et al. 2009) with a rising prevalence currently of 7.6% in Scotland (Norman et al. 2009)and 9.6% of births worldwide (Martin et al. 2006).

Labour is a complex process, with a multitude of different systems and tissues combining and interacting to achieve an elegantly choreographed sequence of events including the remodelling and dilatation of the cervix, rupture of the fetal membranes and the onset and maintenance of effective uterine contractions, culminating in delivery of the fetus and placenta. The entire process is followed by involution of the uterus. In recent years, a growing body of evidence has been published to support the concept that human labour is overwhelmingly an inflammatory process. A better understanding of the physiological processes of normal and abnormal parturition may help us to develop strategies to treat the pathologies associated with childbirth and reduce both maternal and neonatal mortality and morbidity.

In recent years, it has become apparent that the resolution of inflammation, far from being a passive process as was traditionally thought, is an active process and that important mechanisms exist to bring about appropriate resolution of an inflammatory response and reduce host tissue damage. A number of novel mediators have been identified with potent anti-inflammatory and pro-resolution activity.

The following introduction will describe the process of inflammation, its resolution and some of the mediators involved in these processes, with particular regard to those which are relevant to the rest of this thesis. A discussion of the inflammatory processes of labour then follows.

1.2. THE IMMUNE SYSTEM, INFLAMMATION AND ITS RESOLUTION

1.2.1. The immune system

The immune system evolved as a method of defence against infectious disease and tissue injury. A deficient immune system renders the individual susceptible to disease from early life and thus a robust response to infection is vital for survival. However, an immune system that too readily responds to insult may also be damaging to the individual, with the potential to cause tissue damage and/or chronic illness (Nathan 2002). The human body possesses some non-specific defences to protect itself from attack from infectious agents, essentially provided by mechanical barriers such as skin and respiratory tract cilia, secretory factors such as mucus, tears, gastric acid, and the development of normal flora of, for example, the gastro-intestinal tract and vagina (Kumar et al. 2005). However, an effective response must be generated should these defences be breached, and this is provided by what has traditionally been divided into two interacting components: the innate immune system and the adaptive immune system.

1.2.1.1. The adaptive immune system

The adaptive immune response is provided by cells of lymphoid lineage, namely T-cells and B-cells (Kumar et al. 2005). It requires recognition of the antigen by antigen-presenting cells and T-lymphocytes followed by elimination of the antigen by antibodies, effector T-lymphocytes and also by recruitment and enhancement of aspects of the innate immune system (Medzhitov and Janeway 1997). Although the adaptive immune system is able to remember previous initial specific responses and respond more quickly and efficiently during subsequent exposures, its response to infection takes time to evolve and act on the invading pathogens. In contrast, the innate immune system is a non-specific, but rapid response to microbial infection, allowing time for the adaptive immune system to mount a response.

1.2.1.2. The innate immune system

The innate immune response is an ancient evolutionary development and characteristically provides a rapid and non-specific response. It is responsible for inflammation, a phenomenon which has been recognised for thousands of years. Classically defined by the four cardinal symptoms of redness, swelling, heat and pain, inflammation is a response to microbial challenge or tissue injury by which the host mounts a defence against infection and effects tissue repair and a return to homeostasis. An inflammatory trigger is recognised by the host, inflammatory mediators are produced by the sensors that detected the trigger, and these mediators subsequently act on target tissues to bring about functional changes that allow the tissue to adapt to the noxious conditions (Medzhitov 2008).

1.2.2. Inflammation

1.2.2.1. Inflammation in response to infection

The pathways by which the acute inflammatory response proceeds are complex, varied, and differ depending on such factors as the stimulus and the site of insult. Acute inflammation evoked by bacterial infection is perhaps the pathway which has been best characterised, and is briefly described here. Receptors of the innate immune system are able to identify certain components of microbes such as lipopolysaccharide (LPS) and thus are termed pattern-recognition receptors (PRRs) (Medzhitov and Janeway 2000). An example of the PRRs are the Toll-like receptors (TLRs), transmembrane proteins expressed by tissue-resident macrophages, neutrophils and dendritic cells (DCs) (Beutler and Poltorak 2000). Since their discovery in the 1990s (Lemaitre et al. 1996; Poltorak et al. 1998), a multitude of TLRs have been discovered and the scientists that first discovered the TLRs, Bruce Beutler and Jules Hoffmann, were awarded the The Nobel Prize for Physiology or Medicine 2011. Recognition of the specific features of microbes by the TLRs results in activation of the cells on which they are expressed and production of inflammatory cytokines such as interleukin (IL)-1, IL6 and tumor necrosis factor (TNF)-α (Beutler 2002). These inflammatory mediators act upon blood vessel endothelium to promote the migration of leucocytes and the leakage of plasma from within the blood vessels to the site of infection.

Neutrophils, attracted to the inflammatory site, encounter and phagocytose bacteria and then expose the phagosomes to proteases contained in cytoplasmic granules, thus killing and degrading the phagocytosed material (Nathan 2006). Neutrophils can also release the contents of these granules to the extracellular space without having engulfed any bacteria, thus creating a toxic environment for any nearby pathogens. The granular contents are also damaging to host cells and neutrophilic degranulation causes liquefaction of tissue and the formation of pus. This seemingly damaging by-product of inflammation actually serves a beneficial purpose, by containing a bacterial infection in the early phase (Nathan 2006). Neutrophils become apoptotic and are cleared from the site of infection by macrophages, which engulf the apoptotic cells and then leave the site of inflammation via the lymphatic system (Kumar et al. 2005).

1.2.2.2. Sterile tissue injury

Tissue injury is often also associated with concomitant infection, unsurprisingly when the protective barrier of the skin has been broken and microbes are able to invade. However, some tissue injury is sterile, for example, following surgical incision, ischaemic or ischaemia-reperfusion injury. Although the signals and receptors of initiation of inflammation in sterile injury are less well characterised in comparison to those in infective inflammation, it is thought that molecules released from necrotic cells such as heat shock proteins (Vabulas et al. 2002), hyaluronan (Jiang et al. 2005)and β-defensin may be initiators and that the TLRs may be implicated in the recognition of sterile injury (Biragyn et al. 2002). As with infection-induced inflammation, there is recruitment of neutrophils and release of inflammatory mediators. The inflammation acts to help prevent secondary infection and promote repair of damaged tissue.

1.2.3. The resolution of inflammation

Regardless of the original stimulus of inflammation, it is important that inflammation resolves once the stimulus has been dealt with, or host tissue damage or chronic disease may follow. It used to be thought that the resolution of inflammation was a passive process and that once a microbial invasion had been satisfactorily neutralised

and tissue repair completed that the chemoattractants and invading inflammatory response simply 'fizzled out' and the tissues gradually returned to their normal function. However, it is now recognised that active processes exist to control the course of inflammation and promote its timely resolution, thus limiting damage to host tissues (Serhan and Savill 2005). Indeed, the mechanisms by which resolution occur are initiated even as the acute inflammatory response itself is beginning (Serhan and Savill 2005).

These mechanisms include the suppression of pro-inflammatory gene expression, leukocyte migration and activation, followed by clearance of the inflammatory cells by apoptosis and phagocytosis. For example, the TLRs, described above as potent sensors of pathogen invasion, not only induce a pro-inflammatory cascade, but also initiate what ultimately become negative-feedback inhibitors (Liew et al. 2005). Stromal cells at the site of inflammation such as fibroblasts contribute by normalising chemokine gradients and withdrawing survival signals, thus promoting leukocyte apoptosis or exit from the inflammatory sites via the lymphatic system (Filer et al. 2006). One of the most important mechanisms is the development of 'stop signals', which reduce the infiltration of polymorphonuclear neutrophils (PMNs) and promote the phagocytosis of apoptotic cells, and these stop signals include the lipoxins, resolvins and protectins (Serhan et al. 2008), and D-series prostaglandins (Pons et al. 1994; Rajakariar et al. 2007). A phenomenon known as lipid-mediator class switching is observed in which the production pathways of pro-inflammatory eicosanoids such as leukotriene B4 (LTB4) from arachidonic acid (AA) are diverted away towards the production of pro-resolution mediators in the form of the lipoxins (Levy et al. 2001).

As described above, the inflammatory response and its resolution is mediated by a host of different agents including, but not confined to, cytokines, chemokines, prostaglandins, leukotrienes and lipid mediators. Examples of mediators particularly relevant to pregnancy, parturition, the resolution of inflammation and to this thesis are discussed below.

1.3. PRO-INFLAMMATORY MEDIATORS OF INFLAMMATION

1.3.1. Cytokines

Cytokines are soluble proteins or glycoproteins that modulate the functions of other cell types and act to mediate intra-cellular communication. They operate in a complex interacting network, being regulated by a multitude of exogenous and endogenous signals, including each other, and have pleiotropic effects (Hibbert and Johnston 2001). There are a very large number of cytokines, some of which are pro-inflammatory and some of which have anti-inflammatory effects. Examples of cytokines which have particular relevance either to parturition or to the work in this thesis are discussed below.

IL-1

IL-1 is a pro-inflammatory cytokine which exists in two forms, IL-1α and IL-1β. Similar in both structure and function, they also both act upon the two IL-1 receptors, types 1 and 2 (IL1-R1 and IL1-R2) (Sims and Smith 2010). IL-1 is secreted by a large number of cell types, but particularly by activated macrophages, and secretion may be activated by a multitude of triggers including LPS and other cytokines such as tumour necrosis factor alpha (TNF-α). Its potent pro-inflammatory effects include neutrophil chemotaxis, activation of macrophages and epithelial cells, promotion of the proliferation and differentiation of T- and B-lymphocytes and stimulation of other cytokines (Sims and Smith 2010).

TNF-α

TNF-α is a pro-inflammatory cytokine which, like IL-1, is secreted by a multitude of different cell types and has powerful pro-inflammatory effects on a wide range of different cell targets. Its secretion is induced by many factors including bacteria, LPS, IL-6 and IL-1. Effects include neutrophil chemotaxis, and promotion of the production of endothelial adhesion molecules and other pro-inflammatory cytokines (Kumar et al. 2005).

IL-6

IL-6 is a predominantly pro-inflammatory cytokine which, along with IL-1 and TNF-α is responsible for fever, one of the four classical signs of inflammation, and regulates other aspects of the acute phase reaction. It is released by and acts upon many different cell types and is thus implicated in a number of physiological and pathological inflammatory processes (Heinrich et al. 2003). Again, like IL-1 and TNF-α, it has an important role to play in the process of human parturition.

1.3.2. Chemokines

Chemokines are a large subset of cytokines that are grouped together in view of their structural homology and chemotactic properties in stimulating leukocyte movement and regulating leukocyte infiltration from the vasculature into surrounding tissue. Consisting of between 70-130 amino acids, they are classified into families on the basis of the number and location of their N-terminal cysteine residues. The two main families are CC chemokines, in which the cysteine residues are adjacent, and the CXC chemokines, in which there is one amino acid separating the two cysteine residues. A detailed description of chemokine structure and function is found in Baggiolini et al, 2001. Two chemokines with particular relevance to parturition are discussed below.

IL-8

IL-8 is a CXC chemokine, also termed CXCL8. It is initially generated as a 99-amino acid precursor and following cleavage of the leader sequence, the active 72-amino acid form is formed (Baggiolini et al. 1989). Signalling is via CXCR1 and CXCR2, both of which are G-protein coupled receptors. IL-8 is released by a number of cell types including monocytes, macrophages, neutrophils, fibroblasts and endothelial and epithelial cells. Its major function is on neutrophils, upon which it induces adhesion, chemotaxis, activation and the release of granule contents (Baggiolini 2001).

Chemokine (C-C motif) Ligand 2 (CCL2)

CCL2 is also known as monocyte chemotactic peptide-1 (MCP-1) and has 76 amino acids (Yoshimura et al. 1989). As its alternative name suggests, CCL2 is chemotactic for monocytes (Uguccioni et al. 1995), but also for T-cells (Loetscher et al. 1994) and other immune cells and is involved in macrophage activation (Kohl et al. 2010).

1.3.3. Prostaglandins

Prostaglandins are 20-carbon chain fatty acids synthesised from arachidonic acid, which is a constituent of cell membrane phospholipids and liberated from these stores by the action of phospholipases in the first step of prostaglandin synthesis. Arachidonic acid is then converted to an unstable intermediate prostaglandin, PGG2 and subsequently PGH2 by the action of the cyclo-oxygenase enzymes 1 and 2 (COX1 and COX2). PGH2 is then converted into the various series of metabolically active prostaglandins by specific prostaglandin synthase enzymes to form thromboxane A2 (TXA2), prostaglandin D, prostaglandin E, prostaglandin F and prostacyclin (PGI2) (Kumar et al. 2005). Prostaglandins are metabolised by the prostaglandin dehydrogenases (PGDH-1 and PGDH-2), whose principal location is the lung, but which is also localised to the fetal membrane trophoblast (Cheung et al. 1990).

The prostaglandins signal through a variety of receptors to produce diverse effects in many different biological systems. These include both pro-coagulant (TXA_2) or anti-coagulant (PGEs, PGI_2) effects, bronchodilatation (PGEs) or bronchoconstriction (PGFs) and vasodilatation (PGEs, PGI_2) and vasoconstriction (PGFs and TXA_2). Prostaglandins have powerful immunomodulatory effects which may be either pro-or anti-inflammatory and intricate crosstalk with the immune system via cytokine regulation (for review see (Hansen et al. 1999)). Prostaglandins play a vital role within pregnancy and parturition, with key effects in parturition including influence on remodelling and rupture of the fetal membranes (McLaren et al. 2000) and the induction of cervical remodelling and ripening (Denison et al. 1999).

1.3.4. Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of zinc dependent enzymes. Their main function is to degrade proteins of the extra-cellular matrix, and they are thus involved in a multitude of physiological processes. However, they also cleave some cell surface and pericellular proteins, enabling them to regulate cell behaviour, for example by altering the activation of cell surface receptors (Sternlicht and Werb 2001). MMPs are secreted by a wide range of tissues and production is inducible by a variety of factors including cytokines and growth factors. Tissue inhibitors of metalloproteinases (TIMPs) inhibit their release and activation and also have a role in restricting their function (Baker et al. 2002). Within the context of pregnancy and parturition, MMPs have been demonstrated to have an important role in the extracellular matrix (ECM) degradation of the cervix and fetal membranes (Riley et al. 1999), and this is discussed further below.

1.4. PRO-RESOLUTION AND ANTI-INFLAMMATORY MEDIATORS

1.4.1. The Lipoxins

1.4.1.1. Synthesis

The lipoxins are a class of endogenously produced eicosanoids derived from arachidonic acid that have potent anti-inflammatory and pro-resolution effects. First identified in 1984 by Serhan and colleagues (Serhan et al. 1984; Serhan et al. 1984), three pathways have now been identified for the synthesis of lipoxins in humans.

The first of these pathways takes place at the mucosal surfaces. Arachidonic acid is oxygenated by 15-lipoxygenase (ALOX15) to produce 15S-hydroxyleicosatetreanoic acid (15S-HETE). Neutrophil-derived 5-lipoxygenase (ALOX5) then converts this into lipoxin A4 (LXA4) and lipoxin B4 (LXB4) (Serhan et al. 1984; Samuelsson et al. 1987). LXB4 is a positional isomer of LXA4, and differs from LXA4 by the positions of their hydroxyl groups. This pathway not only produces the lipoxins, but diverts away from the pathway producing pro-inflammatory leukotrienes, the afore-mentioned 'lipid-mediator class switching' process.

The second pathway takes place in peripheral blood in a platelet-neutrophil interaction (Serhan and Sheppard 1990). ALOX5 from the leukocytes converts arachidonic acid to leukotriene A4, which is then released and platelet-derived 12-lipoxygenase (ALOX12) converts LTA4 to LXA4 and LXB4 (Serhan and Sheppard 1990) (See Figure 1.1).

The third pathway takes place in the presence of aspirin. Aspirin acetylation of cyclooxygenase-2 (COX-2) inhibits the formation of prostaglandins and promotes the production of 15R-HETE. This can then be converted by ALOX5 to 15-epimer lipoxin A4 and B4 (15-epi-LXA4 and B4) (Serhan 2005), which are also known as aspirin-triggered lipoxins (ATLs).

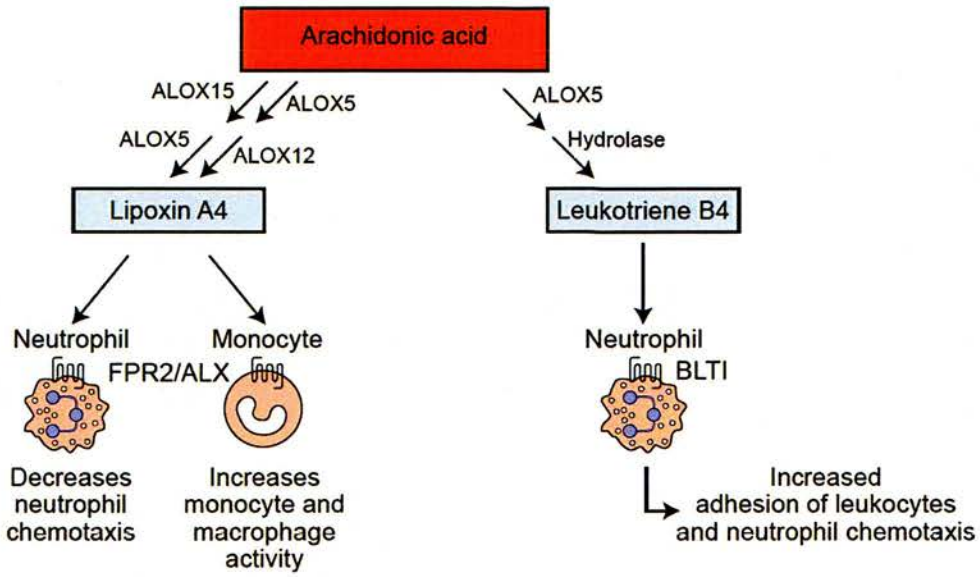


Figure 1.1

Pathways involved in lipoxin A4 synthesis

This figure illustrates the two main pathways involved in the synthesis of LXA4. The ALOX15/ALOX5 pathway takes place in the epithelial cells and neutrophils at mucosal surfaces and the ALOX5/ALOX12 pathway takes place in the blood in a platelet neutrophil interaction. Also illustrated is the pathway involved in the synthesis of Leukotriene B4 which results in pro-inflammatory effects. Figure adapted from Serhan et al 2008

1.4.1.2. The formyl peptide receptors

LXA4 binds with high affinity to a seven-transmembrane domain G-protein coupled receptor, known as the ALX/FPR2 receptor (Ye et al. 2009). This receptor is one of a small family of receptors called the formyl peptide receptors (FPRs). The FPRs are a small family of seven-transmembrane (7-TM) domain G protein-coupled receptors (GPCRs). In humans there are three known members of the family, which share significant sequence homology and which are encoded by clustered genes. Each of the three members have been known by various aliases over time as new data about their structure, function and relationship to each other emerged, but an International Union of Basic and Clinical Pharmacology (IUPHAR)-recommended nomenclature was published in 2009, in which the human FPRs are termed FPR1, FPR2/ALX and FPR3 (Ye et al. 2009).

FPR1

The first member of the family to be characterised was FPR1, with the first reported complementary deoxyribonucleic acid (cDNA) cloning of the receptor published in 1990 (Boulay et al. 1990; Boulay et al. 1990). Initial characterisation of FPR1 described its interaction with N-formylated peptides, which are bacterial peptides with powerful neutrophil chemoattractant properties (Schiffmann et al. 1975). Subsequently, FPR1 has been found to bind with a number of other ligands, including formylated peptides of host mitochondrial origin (Rabiet et al. 2005), non-formylated peptides (Gao et al. 1994) and other peptides, dissimilar to the original bacterial peptides from which the receptor family takes its name. These include an envelope protein of the human immunodeficiency virus type 1 (HIV-1), T20/DP178 (Su et al. 1999), and also Annexin A1 (ANXA1) (Walther et al. 2000) and its N-terminal peptides Ac2-26 (Gavins et al. 2003) and Ac9-25 (Karlsson et al. 2005).

FPR2/ALX

FPR2/ALX was first reported in 1992 by a number of investigators and shown to have significant sequence homology to FPR1 (Bao et al. 1992; Murphy et al. 1992; Perez et al. 1992; Ye et al. 1992). It comprises 351 amino acids, 69% of which are shared with FPR1. Like the other members of the FPR family, FPR2/ALX is a promiscuous receptor, with a wide range of reported ligands. All FPR family ligands reported to date are peptides, with the notable exception of LXA4, an eicosanoid. Reported agonists of FPR2/ALX include N-formyl peptides of bacterial, host mitochondrial and HIV-1 envelope origin, host-derived peptides and LXA4 (for review see Ye et al (Ye et al. 2009)). Ligands of note amongst the host-derived peptides include the chemokine CCL23, an N-terminally truncated splice variant of which is a potent agonist for FPR2/ALX, eliciting the activation of PMNs and chemotaxis (Elagoz et al. 2004). Also of interest are amyloidogenic polypeptides, including serum amyloid A (SAA) (Su et al. 1999), an acute phase protein which induces pro-inflammatory effects including leukocyte chemotaxis (Badolato et al. 1994) and cytokine (Furlaneto and Campa 2000; He et al. 2003) and metalloproteinase production (Vallon et al. 2001). FPR2/ALX, like FPR1, is also activated by the N-terminal peptides of ANXA1 (Perretti et al. 2002), although it appears that the full-length protein, ANXA1, signals through FPR2/ALX, but not FPR1 (Walther et al. 2000).

As shown in the brief discussion above of the multitude of ligands for FPR2/ALX, this receptor is capable of effecting both pro- and anti-inflammatory effects, depending on the ligand and system in question. Indeed, most of the peptide agonists for FPR2/ALX stimulate proinflammatory activities, whereas LXA4, the eicosanoid with a high-affinity for FPR2/ALX, produces powerful anti-inflammatory and pro-resolution effects. It is likely that alternative downstream signalling pathways exist depending on the binding ligand, and these mechanisms remain an area for future study. Competition between ligands for binding of FPR2/ALX is also an area of research that is, as yet, incompletely understood but it may be that preferential binding of LXA4 may contribute to its anti-inflammatory properties by blocking binding by pro-inflammatory agonists.

FPR3

FPR3 is a 352 amino acid 7-TM receptor which shares 56% sequence homology with FPR1. It too binds with some N-formyl peptides, and host-derived peptides, notably F2L, a peptide resulting from cleavage of human heme-binding protein, which activates FPR3 and promotes chemotaxis (Migeotte et al. 2005).

1.4.1.3. Activity of lipoxins

The lipoxins and ATLs have dual anti-inflammatory and pro-resolution effects, which have been demonstrated both in vitro and in vivo and in a number of disease models.

Anti-inflammatory and pro-resolution actions

LXA4 and LXB4 have an inhibitory and thus anti-inflammatory effect on PMNs. PMNs are attracted to sites of inflammation, adhering to vascular endothelial cells and transmigrating across epithelial cell layers in order to access the site of inflammation. LXA4 attenuates nuclear factor kappa B (NF-κB) accumulation and IL-8 expression in neutrophils (Jozsef et al. 2002), can stop neutrophil-epithelial and endothelial cell interactions (Papayianni et al. 1996; Filep et al. 1999), and overall has an inhibitory effect on PMNs, having been demonstrated to reduce chemotaxis, adherence and transmigration (Serhan et al. 1995) and therefore exerting a 'braking effect' on PMN-induced tissue injury.

Whereas the lipoxins inhibit PMNs, their effect on monocytes/macrophages is to activate and promote their actions, thus producing an overall pro-resolution effect. LXA4 stimulates monocyte chemotaxis, migration and adhesion (Maddox and Serhan 1996; Maddox et al. 1997), increasing the recruitment of monocytes to the inflammatory exudate. Subsequently, LXA4 stimulates macrophages, promoting the non-phlogistic phagocytosis of apoptotic neutrophils (Godson et al. 2000; Mitchell et al. 2002) and enhancing the exit of macrophages from the inflammatory exudates via the lymphatic system (McMahon et al. 2001).

Models of disease

Animal models of disease have been used to demonstrate the actions of the lipoxins and epi-lipoxins in vivo in a variety of systems including the lung, kidney, gut and eye, and in models of acute inflammation such as peritonitis or dermal inflammation. In the lung, in a mouse model of asthma, LXA4 inhibited airway hyper-responsiveness and pulmonary inflammation (Levy et al. 2002). LXA4 has been found to be present in reduced concentrations in the bronchoalveolar lavage of patients with cystic fibrosis (CF), and in a mouse model of CF, administration of LXA4 suppressed neutrophilic inflammation, pulmonary bacterial burden and reduced disease severity (Karp et al. 2004). In the kidney, transfection of the ALOX15 gene into rats in whom glomerulonephritis was then induced, resulted in decreased neutrophil recruitment, leukocyte rolling and adherence (Munger et al. 1999). In the bowel, mouse models of colitis have been used to demonstrate that LXA4 attenuates proinflammatory gene expression, reduces the severity of the disease and inhibits weight loss and inflammation (Gewirtz et al. 2002; Fiorucci et al. 2004). LXA4 accelerates corneal re-epithelialisation and limits the effects of thermal injury in the mouse (Gronert 2005; Gronert et al. 2005). LXA4 also reduces hind-limb ischaemia-reperfusion lung injury in mice (Chiang et al. 1999), and LXB4 and 15-epi-LXA4 reduces neutrophil recruitment in dermal inflammation (Takano et al. 1998).

1.4.2. Annexin A1

Annexin A1 (ANXA1) is a 37kDa protein. It is a member of a family of proteins, 'The Annexins' which are grouped together in view of their shared similarity in structural characteristics. Each annexin consists of a core region, made up of four repeats of 70 amino acids each, attached to a unique N-terminal region (Gerke and Moss 2002), which in ANXA1 is 42 amino acids in length (Weng et al. 1993). Upon binding with calcium ions, ANXA1 undergoes a conformational change such that the N-terminal region, previously buried within the core, becomes available for binding, thus generating an active form of the protein (Rosengarth and Luecke 2003). Two short peptides, Ac2-26 and Ac9-25, which correspond to the ANXA1 N-terminal sequence and which have similar actions to ANXA1 itself, have also been identified. The peptides signal through both FPR1 and FPR2/ALX, whereas the full length

protein ANXA1 signals through FPR2/ALX but not FPR1 (Walther et al. 2000; Hayhoe et al. 2006). ANXA1 was first reported over two decades ago (Di Rosa et al. 1984) and was identified as a key modulator of glucocorticoid-mediated anti-inflammation (Fetalvero et al. 2008). Previously known as lipocortin 1, it is now recognised to be a member of the annexin family and termed Annexin A1.

1.4.2.1. Distribution

ANXA1 expression in the human body is widespread, and found in white blood cells, stromal cells and in biological fluids. High levels are present in monocytes and granulocytes, particularly neutrophils, where it is mainly stored within gelatinase granules in the cytoplasm (Perretti et al. 2000). T cells also express ANXA1, but B cells and platelets do not (Morand et al. 1995). Epithelial cells of the lung, gut and kidney are notable sources of ANXA1. In the human reproductive tract, ANXA1 is expressed in the amnion (Lynch-Salamon et al. 1992), chorio-decidua (Myatt et al. 1992) and placental tissue (Bennett et al. 1994), as well as endometrial glandular epithelium (Li et al. 2008).

Location within the cell depends on the cell type. As previously stated, neutrophilic ANXA1 is predominantly cytoplasmic within gelatinase granules; within the macrophage and many other cell types, expression is generally cytoplasmic but has also been noted within the cell membranes, cytoskeleton and nucleus. ANXA1 does have an intracellular role, but it can also be exported out of the cell where it may have autocrine or paracrine function. A number of mechanisms of secretion are reported, namely via activation of the ATP-binding cassette (ABC) transporter (Wein et al. 2004), through phosphorylation followed by membrane localisation (Solito et al. 2006) or (in neutrophils) by fusion of the ANXA1-containing granules with the plasma membrane.

1.4.2.2. Modes of action of ANXA1 and its N-terminal fragment peptides

ANXA1 and its N-terminal fragment peptides have potent anti-inflammatory and pro-resolution effects, which have been demonstrated in a number of experimental models, including in vitro studies with human cells and tissue, animal models of acute and chronic inflammation and in the Annexin A1 null mouse. Perhaps best

characterised are ANXA1's inhibitory effects on PMN trafficking, effecting a reduction in PMN adhesion (Lim et al. 1998; Gavins et al. 2003; Hayhoe et al. 2006) and migration (Errasfa and Russo-Marie 1989; Perretti et al. 1993; Perretti and Flower 1993; Getting et al. 1997; Walther et al. 2000). ANXA1 or its related short peptides also reduce mast cell degranulation (Bandeira-Melo et al. 2005) and increase the phagocytosis of apoptotic cells by macrophages (Arur et al. 2003). They have also been shown to have effects on the expression of other inflammatory mediators, with an inhibitory effect on phospholipase A2 activity (Flower 1988) but a stimulatory effect on the secretion of the anti-inflammatory cytokine IL-10 (Ferlazzo et al. 2003).

ANXA1 has been identified as an important mediator of the effects of glucocorticoids (GCs). GCs induce expression of ANXA1, as reported by Goulding et al, who demonstrated upregulation of ANXA1 in the peripheral blood of human volunteers who had been given hydrocortisone (Goulding et al. 1990). Further evidence is supplied by work demonstrating that individuals with endocrine disorders of either constitutionally high or low levels of endogenous GCs have correspondingly high or low levels of intracellular ANXA1 (Mulla et al. 2005), although the exact molecular mechanism of GC-regulation of ANXA1 is not yet clear (Perretti and D'Acquisto 2009). Additionally, GCs have also been shown to induce the expression of the receptor of ANXA1, FPR2/ALX (Sawmynaden and Perretti 2006; Hashimoto et al. 2007).

1.4.3. The Resolvins and Protectins

Resolvins are endogenous lipid mediators derived from the omega-3 polyunsaturated fatty acids (PUFAs) eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA), resulting in two chemically unique structural forms, the E-series of resolvins and the D-series of resolvins respectively. Resolvins were first identified in exudates that had been collected from resolving inflammatory sites in mice (Serhan et al. 2000) and were subsequently defined and their biological actions described by Serhan and colleagues (Serhan et al. 2002). The consumption of omega-3 PUFAs, found especially in oily fish, had long been regarded as an important part of a healthy diet and had been shown to have beneficial effects on health particularly on

cardiovascular disease and stroke (Simopoulos 2002). However, this was the first time that the molecular basis behind these dietary effects had been elucidated.

Resolvins can be synthesised from EPA via the actions of COX2 and ALOX5 and acts upon the receptors Chemokine-Like Receptor 1 (CMKLR1) and the leukotriene B4 receptor BLT1 (Arita et al. 2007), both of which are GPCRs. Both the D- and E-series of resolvins have potent effects on the reduction of neutrophil recruitment (Serhan et al. 2002; Schwab et al. 2007) and have been shown to control inflammation and promote healing following inflammatory disease in numerous animal models including periodontitis (Arita et al. 2005; Hasturk et al. 2007) and kidney ischemia-reperfusion injury (Duffield et al. 2006).

Protectins are another recently identified family of pro-resolution lipid mediator derived from DHA. Protectin D1 has potent protective properties in inflammatory systems, by inhibiting neutrophil recruitment (Schwab et al. 2007) and regulating cytokine production (Marcheselli et al. 2003) and macrophage function (Duffield et al. 2006).

1.5. LABOUR AS AN INFLAMMATORY EVENT

Human parturition can be regarded as an inflammatory event and in recent years there have been many new additions to the body of evidence that have helped this concept to strengthen and evolve. At its most basic level, this concept is supported by the demonstration that leukocytes, (largely neutrophils and macrophages, and also T cells) invade the myometrium, cervix and fetal membranes at or immediately after the onset of labour (Thomson et al. 1999; Osman et al. 2003), attracted by increased tissue expression of chemokines and cell adhesion molecules (Winkler et al. 1998; Ledingham et al. 2001). In parallel, leukocytes circulating in the peripheral blood increase their expression of cell adhesion molecule ligands such as CD11a and CD11b (Yuan et al. 2009), facilitating leukocyte emigration into the myometrium and cervix.

The increased expression of cytokines in the myometrium and cervix in labour (Osman et al. 2003) arises in part from the invading leukocytes (Young et al. 2002) with recent evidence suggesting that increased capacity of leukocytes in labour to express cytokines is initiated in the peripheral circulation (Yuan et al. 2009). These pro-inflammatory cytokines have a multitude of effects on the reproductive tissues, including stimulatory effects on myometrial contractility via a number of different mechanisms, discussed below. Cytokines are also involved in tissue remodelling (Sennstrom et al. 2000) and attract more leukocytes in a positive feedback mechanism (Elliott et al. 2000), augmenting the process of parturition. The activity of NF-κB, a transcription factor regulating numerous genes involved in a multitude of cellular processes including inflammation, is also increased in labour (Allport et al. 2001). The most clearly described increase in NF-κB in association with parturition occurs in the fetal membranes (Allport et al. 2001), although NF-κB is also expressed in the myometrium with obvious changes being described in pregnancy (Chapman et al. 2004). NF-κB may provide a feed forward mechanism for inflammatory processes in labour, given that it is itself highly inducible by pro-inflammatory stimuli (Belt et al. 1999; Kniss et al. 2001; Lappas et al. 2002).

Taken together therefore, labour is associated with leukocyte invasion, NF-κB activation and pro-inflammatory cytokine production in some if not all of the relevant uteroplacental tissues. These data strongly support the hypothesis that labour is an inflammatory event. The functional effects of these pro-inflammatory events are described below.

1.5.1. Myometrium

The upregulation of pro-inflammatory cytokines within labouring myometrium stimulates and potentiates uterine contractions. Tribe et al have demonstrated that IL-1β induces basal and store operated calcium entry in myometrial smooth muscle cells, thus directly increasing their contractile potential (Tribe et al. 2003). Additionally, IL-1β and TNF-α stimulate arachidonic acid release, and expression of COX-2 thus increasing prostaglandin production in myometrial cell cultures, via greater NF-κB activity (Hertelendy et al. 1993; Molnar et al. 1993; Belt et al. 1999; Rauk and Chiao 2000). Prostaglandins have long been known as stimulators of myometrial contractions (Johnston et al. 1993) thus there is a clear potential causal link between the upregulation of pro-inflammatory cytokines observed in the myometrium and the clinical process of parturition.

1.5.2. The cervix

Cervical ripening is characterised by breakdown of collagen and remodelling of the connective tissue element of the cervix. Leukocyte invasion and the increase in protein concentrations of IL-8, IL-6 and granulocyte colony stimulating factor (G-CSF) observed in the human cervical connective tissue at term and subsequently in labour are likely to be crucial to the process of cervical remodelling to facilitate opening during parturition (Sennstrom et al. 2000; Osman et al. 2003). Functionally, IL-8 may stimulate the release of degradative enzymes from neutrophils. These enzymes include serine proteases and matrix metalloproteinases ((MMP)-8 and MMP-9)) (Kelly 2002) which then participate in degradation of the collagen and glycosaminoglycan components of the extracellular matrix thereby leading to cervical ripening (Hertelendy and Zakar 2004). Production of MMP-1, MMP-3 and MMP-9 from resident fibroblasts (Ito et al. 1991) and smooth muscle cells (Watari et

al. 1999) may also be increased by IL-1β in the cervix whilst downregulating the expression of tissue inhibitor of metalloproteinase (TIMP)-2, an endogenous inhibitor of MMP-2 (Watari et al. 1999), with these events being further amplified by NF-κB production. NF-κB itself may be activated via TLR-4 by degraded extracellular matrix proteins such as fragments of fetal fibronectin, degraded during cervical remodelling, thus reinforcing the ongoing sterile inflammation of labour (Challis et al. 2009). Thus, at the end of pregnancy and during labour, the cervix having remained firm and closed despite increasing pressure during pregnancy, begins, under the influence of a host of inflammatory mediators, to soften and dilate.

1.5.3. Fetal membranes

A similar pro-inflammatory pattern to that observed in the myometrium is seen in the fetal membranes. The production of IL-8, TNF-α, IL-6 and IL-1β are all increased in the fetal membranes and amniotic fluid during labour (Young et al. 2002). There are also increased levels of MMP-9 (Vadillo-Ortega et al. 1995) and decreased levels of the TIMPs (Riley et al. 1999). Functionally, an increased MMP /TIMP ratio weakens the fetal membranes, facilitating fetal membrane rupture which is often the precursor to labour.

Careful examination of the fetal membranes from pre-labour specimens obtained at caesarean section has demonstrated that a zone of weakness exists in the region overlying the cervix (McLaren et al. 1999). The features of this zone include a decrease in thickness in comparison to the rest of the fetal membranes, increased MMP-9 and decreased TIMP-3 and also increased poly (ADP-ribose) polymerase -1 (PARP1) cleavage (El Khwad et al. 2005). PARP1 is an enzyme involved in DNA repair and apoptosis and thus its increase indicates increased apoptosis (El Khwad et al. 2005). It would seem therefore that towards the end of pregnancy the fetal membranes are prepared for labour by a process of remodelling to develop a weakened area that is the site of rupture. What initiates and develops this process is not known but a greater understanding of the molecular events leading to membrane rupture may lead to developing novel therapeutic strategies to halt its premature onset in preterm labour.

The fetal membranes are specifically important, as they are the interface between the fetus and the mother, and may transmit pro-labour signals from the baby to the mother's myometrium and cervix. Pulmonary derived surfactant proteins and phospholipids may be key to this: as the fetus matures, increased quantities are produced and so levels increase in the amniotic fluid. These surfactant proteins may well contribute to the inflammatory response that can be observed in the fetal membranes and in the underlying cervix and myometrium (Smith 2007) given that they stimulate COX-2 activity via TLR-4 in mice (Wang and Hirsch 2003) and subsequent prostaglandin E2 production in the amnion. Increased prostaglandin E2 (PGE2) production in the amnion could diffuse through the chorion and decidua to stimulate myometrial cell prostaglandin production and hence contractility (Slater et al. 1999; Smith 2007). The fetus and placenta may also jointly trigger fetal membrane rupture, since prostaglandins, TNF-α and corticotrophin releasing hormone (CRH) (which also rise in the amniotic fluid towards term (Laatikainen et al. 1988; Alvi et al. 1999) all stimulate the production of MMP-9, which weakens the membranes and thus facilitates membrane rupture (Young et al. 2002; Kumar et al. 2006).

1.5.4. The onset of labour, term and preterm

Although much is known about the processes of labour, both term and preterm, what actually is the initiating event that kickstarts human parturition remains something of a mystery. In several animal species, events seem to be much more clear-cut, and indeed we can use our knowledge of other species to gain clues to the human situation. For example, the phenomenon of progesterone withdrawal, first described by Csapo in the 1950s (Csapo 1956), and characterised by a sudden drop in serum progesterone levels just prior to parturition, is a common feature in many mammalian species. However, in humans, most studies have shown that serum progesterone levels remain stable towards the end of the third trimester and during labour (Boroditsky et al. 1978; Mathur et al. 1980). A 'functional progesterone withdrawal' has been described, whereby there is loss of the quiescent effect of progesterone on myometrium, despite continuing high systemic and local levels. There have been

several different mechanisms proposed for this, including local metabolism of progesterone (Mitchell and Wong 1993), changes in ratios of the progesterone receptor isoforms (Mesiano et al. 2002) and changes in levels of cofactors affecting progesterone receptor (PR) function (Condon et al. 2003). It seems that a multitude of complex and interlinked processes combine to overwhelm the quiescent phase of pregnancy and initiate labour. Mitchell and Taggart have suggested a model of 'modular accumulation of physiological systems' in which multiple interlinking physiological processes develop in parallel until a critical mass is achieved which results in parturition (Mitchell and Taggart 2009). Their model also describes how the premature and inappropriate activation of some of the 'modules' could result in preterm labour, thus such a concept could explain the onset of both term and preterm human labour.

Progesterone has long been described as an 'anti-inflammatory steroid' and this is supported by a wealth of data demonstrating its interactions with the immune system. Progesterone itself inhibits IL-6 production in fetoplacental arteries (Gotkin et al. 2006), and progestogens such as medroxyprogesterone acetate inhibit a range of pro-inflammatory cytokines and chemokines in human myometrium (Shynlova et al. 2008; Youssef et al. 2009). Of particular interest are the interactions of progesterone with NF-κB, a transcription factor which has key functions within human parturition (Allport et al. 2001; Elliott et al. 2001). NF-κB activation increases in fetal membranes in labour (Allport et al. 2001) but is negatively repressed by activation of the progesterone receptor (Allport et al. 2001). In contrast, NF-κB itself represses progesterone receptor activity; work from Carole Mendelson's group has demonstrated that activation of NF-κB in human myometrium upregulates inhibitory PR isoforms (Condon et al. 2006). It may be that the removal of the immunosuppressive, quiescent influences of progesterone by the functional progesterone withdrawal may in fact itself be initiated by inflammation and activation of NF-κB (see Fig 1.2).

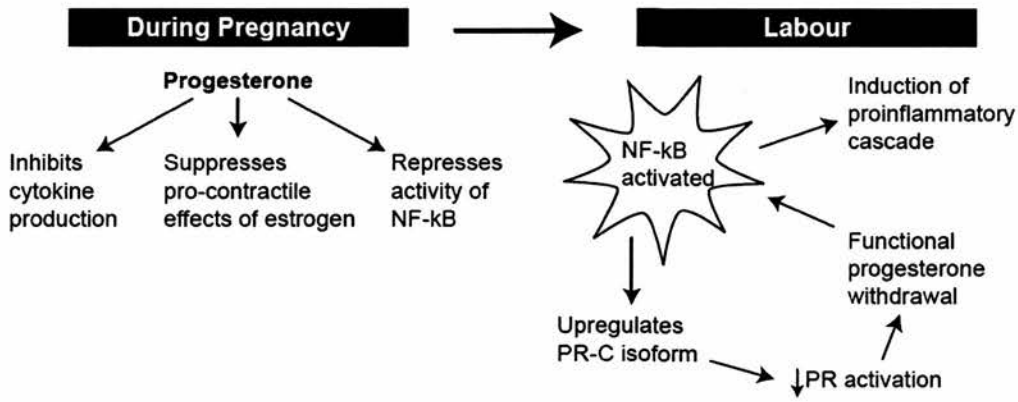


Figure 1.2

Putative Mechanisms of Influence of Progesterone

During human pregnancy, progesterone levels are high and progesterone exerts a quiescent effect on the myometrium.

At the time of labour, increased NF-κB activation decreases PR activation thus further diminishing the anti-inflammatory influences of progesterone, allowing the amplification of the proinflammatory cascade.

Estrogens can induce either proinflammatory or anti-inflammatory effects, depending on the immune stimuli, the cell types involved, the target organ, estrogen concentration and receptor expression (Straub 2007). With respect to cytokine expression, estrogen in the high concentrations seen in pregnancy can inhibit proinflammatory pathways which include those activated by IL-1β (Polan et al. 1989), IL-6 (Keck et al. 1998; Kikuchi et al. 2000), IL-8 (Rodriguez et al. 2002) and TNF-α (Rogers and Eastell 2001) as well as inhibiting the activity of natural killer cells (Seaman and Gindhart 1979). In contrast, the secretion of 'anti-inflammatory interleukins' IL-4 (Kamada et al. 2001), IL-10 (Kanda and Tamaki 1999) and transforming growth factor (TGF)-β (Hatthachote and Gillespie 1999) are stimulated by estrogen in these high levels. Notwithstanding these clear anti-inflammatory effects of estrogen, in vivo estradiol can augment the production of pro-inflammatory cytokines by CD4+ T cells (Maret et al. 2003) likely via a direct effect as circulating leukocytes express estrogen receptor (ER)-α (Straub 2007). Thus, although estrogen has a stimulatory effect on myometrium, it also has highly complex interactions with the immune system that may contribute to the quiescence maintained throughout pregnancy.

CRH and the hypothalamic-pituitary-adrenal (HPA) axis are intimately linked with the immune system and exert complex effects on the human female reproductive system (Kalantaridou et al. 2004). CRH has been shown to regulate cytokine production, and in return inflammatory cytokines influence CRH production. For example both TNF-α and IL-1β stimulate production of CRH (Dudley 1999) and conversely CRH inhibits the production of IL-1 and IL-6 by peripheral blood mononuclear cells (Salas et al. 1997). CRH may reduce the cytotoxicity of natural killer cells (Irwin et al. 1990) and also inhibits T cell proliferation (Jain et al. 1991). Increasing fetal cortisol production, perpetuated by increasing CRH production as described above, is also associated with fetal lung maturation and production of surfactant proteins and phospholipids which are present in the amniotic fluid. Such compounds are proinflammatory and it has been proposed (Smith 2007) that they might stimulate production of prostaglandins and initiation of inflammation in the fetal membranes.

Oxytocin and its receptor (OT and OTR) play an important role in parturition, although their precise role is as yet incompletely understood (Blanks and Thornton 2003). Plasma concentrations of OT rise with labour (Thornton et al. 1992), as do concentrations of OT in gestational tissues (Blanks et al. 2003). Clinically, synthetic oxytocin is used to augment contractions during dystocic labour and OTR antagonists are licensed as tocolytic agents for the treatment of preterm labour (Romero et al. 2000). However, OT appears not to be an essential participant in labour as oxytocin (-/-) null mice can deliver normally (Nishimori et al. 1996; Young et al. 1996). The oxytocin receptor increases at term in myometrial tissue (Fuchs et al. 1984) and its increased expression leads to direct contractile effects in myometrium (Blanks and Thornton 2003). The effect of inflammatory cytokines on the OTR is somewhat conflicting as described in the literature. Schmid et al reported a downregulation of OTR messenger ribonucleic acid (mRNA) by IL-1β in a myometrial cell line (Schmid et al. 2001), and Rauk et al also reported down-regulation of OTR mRNA following treatment with IL-1β and up-regulation after treatment with IL-6 in primary human smooth muscle cells (Rauk and Friebe-Hoffmann 2000; Rauk et al. 2001). However, Terzidou et al demonstrated rapid transient upregulation of OTR mRNA with treatment of primary human myocytes with IL-1β (Terzidou et al. 2006) which is more consistent with the actual mileu present in labour ie increased levels of IL-1β, COX-2 and IL-8. The OTR promoter contains putative transcription binding sites for C/EBP and NF-κB, which are transcription factors activated by cytokines such as IL-1β and IL-6 (Terzidou et al. 2006). IL-1β and IL-6 increase the secretion of oxytocin in uterine smooth muscle cells (Friebe-Hoffmann et al. 2001) and IL-1β has been observed to augment the oxytocin-stimulated contractility of myometrial strips in culture (Molnar et al. 1993). Thus, it seems likely that the result of the infiltration of inflammatory cells and increase in pro-inflammatory cytokines is to activate the myometrial tissue (at least in part via an increase in OTR expression) and to promote contractility.

So if the onset of human labour has features of inflammation and endocrine changes, which is the predominant regulator of parturition? There is little compelling evidence to suggest that in humans a hormonal switch exists that signals the end of pregnancy and begins the process of parturition. It is likely that the linkages between such

processes are dominated by the inflammatory system and that whilst hormones play an undoubtedly vital part in human parturition; it is the immune system that is the more important driving force.

1.5.5. Preterm Birth and Inflammation

Preterm labour is defined as labour occurring before 37 completed weeks of gestation. The clinical implications are profound, as preterm birth is the major cause of neonatal mortality and morbidity in developed countries (Norman et al. 2009). In England, Wales and Northern Ireland, 74% of neonatal deaths are in infants born preterm . Preterm birth has a rising prevalence currently of 5.8% in Scotland (Norman et al. 2009) and 9.6% of births worldwide (Martin et al. 2006). Preterm labour (with or without preterm premature membrane rupture) is the cause of preterm birth in around 75% of women (Norman et al. 2009). Preterm labour may be initiated by a multitude of different mechanisms, including infection, placental haemorrhage, cervical insufficiency, and uterine over-distention. The majority of preterm births are idiopathic (Goldenberg et al. 2008; Norman et al. 2009), however, infection is the single biggest aetiological factor (Goldenberg et al. 2008). Micro-organisms such as Escherichia coli, Ureaplasma urealyticum and Streptococcus agalactiae are recognised by the TLRs, which stimulate increased production of inflammatory cytokines at least in part by NF-κB and initiate the cascade of inflammatory events described above, resulting in preterm parturition (Peltier 2003).

Intrauterine infection can come via various routes, including ascending infection from the vagina, transplacentally, retrograde infection via the Fallopian tubes and by iatrogenic introduction to the intrauterine cavity during invasive procedures such as amniocentesis. Intrauterine infection may be found in the decidua, the space between between the amnion and chorion, in the amniotic cavity or even in the fetus. Clinically, maternal infections such as pyelonephritis, appendicitis and pneumonia are associated with preterm birth (Goldenberg et al. 2008) and periodontal disease has also been relatively recently recognised as being linked to preterm birth (Jeffcoat et al. 2001), although a mechanism of causation is not clear.

Even in the absence of preterm birth itself, intrauterine infection or inflammation is damaging to the fetus. This is demonstrated by clinical and epidemiological studies (Dammann and Leviton 1997; Kadhim et al. 2002; Hagberg et al. 2005) and also by animal models (Elovitz et al. 2011). This factor complicates the development of treatments for preterm labour because it means developing a method of keeping the fetus in utero (thus avoiding prematurity) is not necessarily going to be beneficial to the infant. The fetus must also be protected from the detrimental effects of inflammation and infection that may be present during threatened preterm birth.

1.5.6. Animal models of preterm birth

Several animal models of parturition involve stimulation with pro-inflammatory substances such as LPS or bacteria to mimic the onset of preterm labour. These include rodent models such as rat (Terrone et al. 2001; Celik and Ayar 2002), and mouse models (Fidel et al. 1994; Elovitz et al. 2003), and also primate models using rhesus monkeys (Gravett et al. 1994). Such animal models may be used to develop potential new strategies for treating preterm birth. For example, the importance of inflammation is demonstrated by experiments showing that the anti-inflammatory agent IL-10 not only prevents preterm birth, but also prevents neonatal brain damage in rats (Terrone et al. 2001; Rodts-Palenik et al. 2004). More recently, Bennett's group have shown that the anti-inflammatory prostaglandin 15-deoxy-delta(12,14)-prostaglandin J(2) (15d-PGJ(2)) may have similar dual effects: in their mouse model 15d-PGJ(2) inhibited both LPS induced contractions and preterm delivery and the upregulation of NF-κB in the fetal brain (Pirianov et al. 2009). These results encourage the possibility of the future development of agents that could be used not only to prevent preterm labour but also to protect the developing fetus from the damaging effects of surrounding inflammation.

1.6. PRO-RESOLUTION MEDIATORS IN PARTURITION

Inflammatory regulation is a key feature of many physiological processes within the human reproductive tract (Jabbour et al. 2009), and indeed, evidence is emerging that pro-resolution mediators have an important role to play within these processes. For example, in the endometrium, it would seem likely that inflammatory resolution is involved in the monthly cycle of cell proliferation, disintegration, shedding and tissue repair, and indeed both ANXA1 and FPR2/ALX have been implicated in this process (Gurpide et al. 1986; Macdonald et al.2011). Additionally, ANXA1 would appear to be involved in the pathological process of endometriosis (Li et al. 2008) and stable LXA4 analogues have shown promising resolution effects on endometriotic lesions in a mouse model of endometriosis (Chen et al. 2009; Chen et al.).

Despite the well-established and supported concept of parturition being an inflammatory process, surprisingly little has yet been published on the potential role of pro-resolution mediators such as LXA4 and ANXA1 in pregnancy and parturition. ANXA1 is known to be upregulated in pregnant compared to non-pregnant tissues (Romisch et al. 1992; Rehman et al. 2003) but a functional role has yet to be determined. Our laboratory has recently published work describing an upregulation and potential role of LXA4 in early pregnancy (Macdonald et al. 2011) but little is known about its role in later pregnancy and during parturition. It would appear that there is much to explore for the role of these pro-resolution mediators within pregnancy and parturition itself.

1.7. CONCLUSIONS

This introductory chapter has considered the process of inflammation and its resolution and described some of the mediators involved in these processes that are particularly relevant to parturition and the work presented in this thesis. It has also discussed the inflammatory events of human labour, both term and preterm and speculated on the potential roles of pro-resolution mediators within these events.

The biochemical mechanisms of human parturition in which the membranes weaken and rupture, the cervix softens and effaces and the uterus is activated to produce contractions, are both highly complicated and incompletely understood. It is clear that an intricate web of interactions exists between components of the endocrine and immune systems in order to regulate this process, and that disorders within it may lead to significant complications for both mother and infant. As knowledge accumulates about the mechanisms of preterm and term human labour, we can hope to come closer to effective therapies for preventing preterm or dysfunctional labour, and providing a healthier environment for the baby should intrauterine inflammatory pathways be activated prematurely.

Recent advances in the exploration of the inflammatory process of parturition and the potential role of pro-resolution mediators have been highly encouraging. It seems likely that in the future a multi-pronged approach will be necessary to treating preterm or abnormal term labour and there is much scope for drugs developed from pro-resolution mediators forming a part of that approach, with potential effects not only on the inflammatory process of labour, but also in manipulating the detrimental effects of inappropriate inflammation on the fragile fetal tissues. It is hoped that with further investigation of the role of pro-resolution mediators in human parturition, we may find new ways of treating preterm or abnormal term labour and reduce the burden of these disorders on women, their children and the health systems that support them.

HYPOTHESIS AND AIMS

Pregnancy is a state of relative inflammatory suppression, culminating in parturition, which is characterized by a profoundly pro-inflammatory environment within the myometrium. This concept may be thought of as a 'balance', in which some factors act to promote the quiescent state of pregnancy and others act to promote the inflammation associated with labour. During healthy pregnancy this 'balance' is tipped in favour of ongoing pregnancy, until such time as the fetus is mature enough to be born, when the 'balance' tips the other way and labour ensues.

I hypothesised that the onset of parturition is influenced not only by pro-inflammatory mediators, but also pro-resolution and anti-inflammatory mediators, specifically LXA4 and ANXA1, and that their expression would decrease during labour. Furthermore, I hypothesised that stimulation of non-labouring reproductive human tissue by stress mediators such as lipopolysaccharide, hypoxia and cortisol would result in a rise in pro-resolution/anti-inflammatory mediators, in order to attenuate the effect of the external stimulus and maintain the quiescent state of pregnancy.

My aims were:

1. To investigate the mRNA and protein expression of pro-resolution/anti-inflammatory: LXA4 (and its enzymatic inducers, the lipoxygenases), ANXA1, and their mutual receptor FPR2/ALX in pregnant human labouring and non-labouring reproductive tissue.

2. To investigate the overall transcriptomic response of pregnant human non-labouring myometrium to pro-inflammatory LPS and pro-resolution/anti-inflammatory LXA4.

3. To investigate the mRNA and protein expression of pro-resolution/anti-inflammatory LXA4 (and its enzymatic inducers, the lipoxygenases); ANXA1; and their mutual receptor FPR2/ALX in pregnant human non-

4. To investigate how cortisol, a hormone associated with the promotion of labour, may affect the production of ANXA1 and its receptors in human pregnant myometrium.

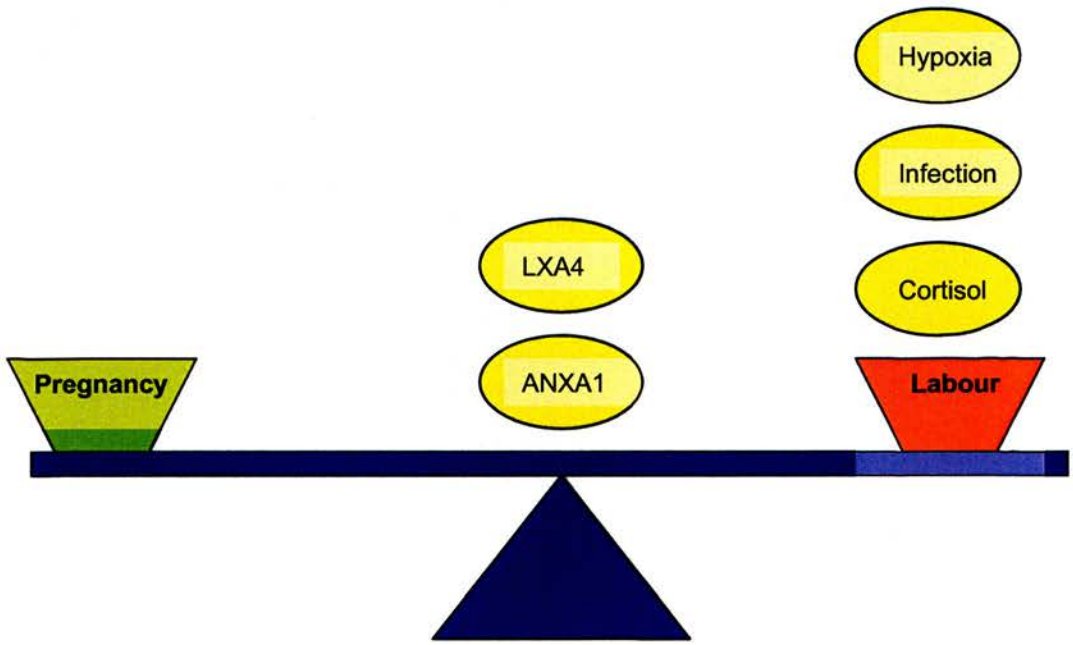


Figure 1.3

Diagrammatic representation of hypothesis

This figure represents the 'balance' existing between ongoing pregnancy, generally thought of as a state of relative immune suppression, and the onset of labour, in which a pro-inflammatory mileu dominates. Cortisol and infection are known to be associated with the promotion of labour. Tissue hypoxia is a pro-inflammatory stimulus. I hypothesised that the pro-resolution and anti-inflammatory effects of LXA4 and ANXA1 promote the continuation of pregnancy and that hypoxia, cortisol and LPS would influence the expression of these mediators.

2. General Methods

2.1. THE COLLECTION OF TISSUE FOR A REPRODUCTIVE TISSUE BIOBANK

2.1.1. Introduction

Biomedical research in reproduction requires volunteers to provide tissue and blood samples upon which experiments can be performed and hypotheses tested. Tissue collection can either be prospective, and tailored towards each individual project, or banked and a resource established from which investigators can apply to have tissue samples supplied for use in their experiments. Each approach has its advantages and disadvantages. Prospective collection has the advantage of providing the investigator with exactly the requirements they have identified as necessary for their project, in terms of patient demographics, specimen type, time of collection, method of collection and method of storage. However, clearly the disadvantage is that the investigator needs to invest time and resources into performing the recruitment and collection themselves, and must wait for the necessary samples to become available, which may take long periods of time. Furthermore, some projects require longitudinal outcome measures to be obtained and again, it may be that it takes a long time to collect this data. In contrast, a tissue bank provides the researcher with a resource from which they can obtain immediate access to a potentially much larger group of samples than they would otherwise have been able to obtain. Samples will have been collected and stored uniformly according to a Standard Operating Procedure (SOP), and will be accompanied by patient demographic data. Tissue Banks also allow for samples to be available to multiple researchers, maximising their use and promoting collaboration. The disadvantage of this approach is that the available specimens may not meet the exact requirements of the project for which they are sought, be that in terms of quantity, method of sampling or method of storage.

In Edinburgh, there has been set up the Edinburgh Reproductive Tissue Biobank to collect and store samples for use by current researchers, by collaborators within the department or university and by collaborators at other institutions. The stored samples are also potentially available for future researchers upon application, both

from our own institution and from others. Part of the work I undertook over the course of my two-year MD programme of study was to recruit patients to contribute tissue for the Biobank, to write and publish SOPs and to organise and train other members of the research team to recruit patients and perform the tissue collection. In this section I will describe the remit and scope of the Edinburgh Reproductive Tissue Biobank, my role within its development, and its current and potential future applications.

2.1.2. The Edinburgh Reproductive Tissue Biobank

The Biobank is funded by the Piggy Bank Kids and by Tommy's charities that funds maternal and fetal research, and by the University of Edinburgh. It is managed by a group of doctors, midwives, scientists and lay members who ensure that the relevant ethical and legal requirements are adhered to and that the tissue is used appropriately. The Biobank comprises a collection of blood and tissue samples from pregnant and post-natal patients who have been recruited from the Simpson's Centre for Reproductive Health at the New Royal Infirmary of Edinburgh. This is a tertiary referral maternity unit delivering over 6500 babies each year. The collection also includes some samples from infants, and a full list of the types of tissue that may be collected is found in the Appendix. The following paragraphs describe the groups of women upon whom collection efforts have so far been focused.

2.1.2.1. Metabolic clinic patients

Much of the funding our laboratory receives is from Tommy's, a UK-based charity that funds research into maternal and fetal health, and a major focus of the team's research is focused on the problems caused by obesity in pregnancy. Clinical members of our team run a Metabolic Antenatal Clinic to which pregnant women booking with a BMI of 40 kg/m^2 are invited to attend to receive antenatal care for the duration of their pregnancy. Women attending the clinic are subsequently invited to participate in various research studies, including the donation of tissue and blood samples at the time of delivery. Women with a normal Body Mass Index (BMI) of between 20 and 25 kg/m^2 are also recruited to the studies as control patients and may also consent to donate blood and tissue samples at the time of delivery. In addition to the blood and tissue samples donated by this group of patients, a large amount of

information is collected and archived during the pregnancies and post-natally. This includes longitudinal data on physical characteristics such as weight, BMI, body fat percentages, skin fold thicknesses and blood pressure; dietary intake during pregnancy, fetal growth trajectories, delivery outcomes and neonatal growth and development. Thus the Biobank stores not only biological material but is also a resource for storing information. These patients provide a unique dataset and valuable tissue samples and represent one of the main foci of collection to the Biobank.

2.1.2.2. Women undergoing elective caesarean section

Many research institutions use the clinical scenario of elective caesarean section as an opportunity to approach women for recruitment of tissue donation. Some tissues, for example, myometrium, fat and ovarian tissue can only be collected during a surgical procedure. A planned operation means there is plenty of time to approach patients and discuss their participation, and the subsequent collection from recruited patients takes place at a convenient and predictable time. Many of the women are at term with uncomplicated and healthy pregnancies, often undergoing caesarean section due to previous caesarean section or breech presentation. This is of value to researchers who wish to use tissue in their experiments from 'normal' pregnancies, either as a control against tissue from pregnancies complicated by the disorder they are interested in, or to exclude any confounding effect that medical or obstetric complications may introduce. In our laboratory, many researchers have required 'fresh' tissue for use in their experiments i.e. tissue that has been collected that day, and these samples are generally collected at elective caesarean section. Alongside such a collection of 'fresh' tissue, a collection of tissue is also made which is stored for RNA, protein and immunohistological experiments. There therefore exists in the Biobank a large collection of stored tissue from women in 'normal' pregnancies that can be used as controls.

2.1.2.3. Labouring women

In researching parturition and preterm labour, the ability to compare tissue from both labouring and non-labouring women is obviously very important, but also can be difficult due to the challenges of recruiting women in labour. The ethics approval of

the Biobank requires us to use our clinical judgement to avoid approaching women who are unsuitable to discuss participation, but allows researchers to approach labouring women, if this is deemed appropriate. Women in early labour are often willing to be approached to consider donation to the Biobank and frequently consent to recruitment. Researchers are thus always guided by the advice of the clinical staff looking after the woman, and their own assessment during discussion, as to whether a woman should be approached for recruitment. Placental tissue can be collected easily after both vaginal and caesarean delivery but the collection of myometrium and fat can only be performed at caesarean section. During labour it can be difficult to predict which patients will end up having an emergency 'in-labour' caesarean section and so the collection of myometrium and fat from labouring women requires the recruitment of large numbers of women to donate samples at delivery, of whom only a certain number will end up having a caesarean section and actually donating myometrium and fat. On the whole, my experience of recruiting women in labour was very positive. Of those women who were willing to speak to a researcher, many were very willing to take part and help by donating tissue and hopefully this part of the collection will provide a valuable ongoing resource and be expanded with time.

2.1.2.4. Other groups of women

There are many other groups of women from whom it is valuable to have a banked collection of tissue. Examples include women with pre-eclampsia and women delivering preterm, both labouring and non-labouring. Identifying such specific patients to approach them for recruitment can be difficult and requires help from the clinical staff looking after them to alert researchers that a suitable patient might be in the hospital.

2.1.3. My role within the Biobank development

My role within the Biobank has been to help develop the process of recruitment and collection of delivery samples, to co-author SOPs, to co-ordinate the team of researchers carrying out daily recruitment at elective caesarean section, and to help with teaching and supervising new members of the collecting team. The Biobank and the samples it contains are an important and valuable part of the research work our laboratory carries out and the whole team is involved in the collection and

administration of its samples. In the work I have described above, I worked alongside other team members and would particularly like to acknowledge the following people for their involvement in the work I carried out: Dr Sarah Barr, Rose Leask, Zillah Jones and Nanette Hibbert with whom I helped to develop and co-author the SOPs by which the samples are collected.

2.1.4. Developing the Standard Operating Procedures

The process of collection of the tissues needs to be straightforward, easy to teach and recall and easily reproducible so that samples are reliably collected and stored in the same way each time, despite being collected by different individuals. In order for this to be achieved, it is important to have robust SOPs. The SOPs used in our collection processes have developed and modified over time and with use, but if earlier samples are requested for use from the Biobank it is possible to describe in what way sample collection or storage may differ from the most up-to-date SOP, if applicable.

The SOPs we have produced are designed to be easy to use and possible to refer to whilst actually performing the collection. A description of the methods used to collect the tissue samples that are routinely collected at delivery, and how they are stored in the Biobank, follows in the subsequent paragraphs.

2.1.5. Tissue sampling and storage

2.1.5.1. Methods of harvesting tissue samples at delivery

The following tissue samples may be collected at delivery:

Myometrium: Following delivery of the baby and placenta and prior to closure of the uterine incision, the operating surgeon incises a strip of myometrium from the upper edge of the lower uterine segment incision using Mayo scissors. The strip is full thickness of the uterine wall and 1cm by 2-3cm in other dimensions. This sample is placed into a sterile Gallipot and handed to the researcher by the scrub nurse. The researcher then dissects off deciduas and divides the sample into pieces that are stored in several different ways to be used in different kinds of experiments, as described below.

Visceral fat: Prior to closure of the rectus sheath, the operating surgeon identifies the omentum, applies clamps across a small section of the tissue, divides this with Mayo scissors and ligates the tissue across the clamps with vicryl ties. The sample is placed into a sterile Gallipot and handed to the researcher, who then divides the sample into the pieces and places them into storage containers.

Subcutaneous fat: Following closure of the rectus sheath and prior to skin closure, the operating surgeon excises a small piece of subcutaneous adipose tissue using Mayo scissors, which is placed into a sterile Gallipot and handed to the researcher, who then divides the sample into the pieces and places them into storage containers. During the team's earlier experiences of tissue collection, surgeons used diathermy to excise a piece of tissue, which had the advantage of excising the sample whilst simultaneously ensuring haemostasis at the excision site. However, subsequent use of the tissue in experiments suggested that this collection technique might be associated with poorer yields of RNA from the sample, presumably due to thermal destruction of the tissue close to the line of incision. Surgeons are now therefore requested to use sharp dissection to produce the sample and then use diathermy to achieve haemostasis.

Placenta, fetal membranes, umbilical vessel blood and umbilical cord: Following delivery of the baby, the cord is clamped and cut. A second clamp is then applied to the umbilical cord to isolate a section of cord from which blood samples may be obtained. This is important clinically as samples are taken from the umbilical vein and artery for pH measurements for the neonatal medical record, and where the mother is Rhesus negative, for determination of the baby's blood group. Midwives and surgeons are encouraged to place the clamps as far apart as possible to leave as long a stretch as possible undamaged by the clamps. If suitably trained the researcher can collect the clinical samples and then collect samples of cord blood for research. The research samples are collected by piercing the umbilical vein or artery with a needle and withdrawing blood into a syringe. If insufficient blood is collected from the umbilical vessels in the cord, blood may be collected from the blood vessels

covering the fetal side of the placenta. Blood is then transferred into Monovette tubes and then transferred to the laboratory on ice for storage.

Small sections of umbilical cord are cut using scissors or a scalpel and placed into storage containers. Lengths of umbilical cord to be used immediately in experiments are transported to the laboratory on ice.

For use in immunohistochemistry, a strip of fetal membrane comprising amnion and chorio-decidua is cut using scissors. The strip is taken from the rupture site to the placental edge and includes a tiny amount of placental tissue to orientate the sample. This sample is stored as detailed below and when at the stage of being embedded in paraffin is rolled into a tight roll of tissue so that when sections are cut the researcher has a representative slice of amnion, chorio-decidua and placental tissue. Samples of amnion and chorio-decidua are also cut using scissors and stored for RNA and protein analysis as described below.

Placental tissue is cut using scissors from the centre of a cotelydon. To ensure continuity within the collection, samples are taken from a cotelydon close to the umbilical cord insertion and from a part of the placenta that does not contain any areas of calcification or large blood vessels. Samples for immunohistochemistry are full thickness including the covering membranes on the fetal side and the basal plate on the maternal side. Other stored samples are taken from the centre of the placenta and do not contain covering membranes or basal plate tissue.

2.1.5.2. Storage of tissue samples for RNA extraction

Samples collected for later RNA extractions are placed into RNAlater solution, incubated at 4°C overnight and then removed from solution, placed into a fresh storage tube and frozen at -80°C. The advantage of using RNAlater is that the RNA in the tissue is stabilised and the expression profile is protected so that subsequent RNA analyses accurately reflect the expression profile at the time of collection. The manufacturers advertise that samples can be stored at 4°C for up to 4 weeks, and in theory could be removed at any time during this period for archival storage.

However, by dealing with all samples in the same way we can ensure consistency in the collection.

The exception to this protocol for RNA collection is for fat samples, both visceral and subcutaneous. RNAlater was not used for these samples as the lipid in the sample prevents adequate penetration of the stabilization agent through the tissue. Thus initially, fat samples for RNA were placed into AllProtect, kept at 4°C until the following day and then transferred to a separate tube and frozen at -80°C. However, upon thawing these samples and extracting RNA, it was found that poor yields of RNA were obtained, and that samples that had been frozen on collection without having been previously incubated in AllProtect actually gave better RNA yields. Thus, the current protocol for collecting and storing fat samples is to place them dry into a collection tube and store them immediately at -80°C.

2.1.5.3. Storage of samples for protein extraction

Samples are collected, put into tubes and immediately placed into storage at -80°C. Initially samples had been snap frozen on dry ice prior to storage at -80°C, but as there is not a source of dry ice in the hospital itself, this became impractical. It was determined that the method of freezing the samples by placing them straight into the freezer was sufficient to allow good yields of protein when subsequently extracted.

2.1.5.4. Storage of samples for histology

Samples are collected, placed into formalin, transported to the laboratory and stored at room temperature overnight. The samples are then placed into ethanol and stored at 4°C. They are then embedded in paraffin and stored ready for use by researchers when required.

2.1.5.5. Collection of samples for immediate use in experiments

As well as tissues being collected for storage and future use, the Biobank set-up allows for the collection of 'fresh' tissue, which can then be used immediately in experiments. Examples of such experiments that have been carried out using tissue collected by our team include fluorescence-activated cell sorting (FACS), explant

culture, myometrial contractility studies and harvesting of human umbilical vein endothelial cells (HUVECs) from umbilical cord. Material obtained from such experiments, for example RNA extracted from explant studies, can then be placed in the Biobank and recorded in its database so that future researchers could also take advantage of this resource. Researchers requiring fresh tissue can apply to the Biobank management team with a request of what they need and then the fresh tissue is collected according to their requirements alongside the standard Biobank collection.

2.1.5.6. 'Out-of-hours' tissue collection

Much of the tissue collected for the Biobank is obtained from women undergoing elective caesarean section, which is a scheduled operation occurring within normal working hours and therefore relatively easy to predict and collect. However, many of the women from whom valuable tissue may be collected do not deliver by elective caesarean section. Instead they may go into labour spontaneously or be induced, and may deliver vaginally or by emergency caesarean section. Examples include women recruited from the Tommy's Metabolic Clinic, both obese and normal-BMI controls; women delivering pre-term either in labour or not in labour; women with pre-eclampsia, and women in labour at term. Clearly these deliveries and thus the opportunity for collecting tissue samples may occur at any time around the clock and not just 9-5 Monday-to Friday. Thus, the Biobank team have recruited a team of medical students who are interested in research and/or Obstetrics and Gynaecology to perform collections on women who have already been recruited to donate samples who deliver 'out-of hours'. The students are trained how to perform the collection and then form an on-call rota, covering 5pm-9am Monday to Friday and all day Sunday. This system, although time-consuming to set up, has been a great success, with many valuable samples being added to the collection that would otherwise have been lost. It has the added advantage of introducing medical students to research, and perhaps sparking their interest to become further involved in their future careers.

2.1.6. Conclusions

The Edinburgh Reproductive Tissue Biobank is a valuable research tool and being involved in its development has been a rewarding and educational part of my MD

project. Many women are very keen to be involved in research during their pregnancy and willing to help researchers. Frequently during the course of speaking to women during recruitment they would describe experiences of adverse maternal or fetal outcomes that they had come across, either in their own previous pregnancies or in friends' or relatives', and express a wish to be involved in our research with the hope of helping to prevent future such outcomes. Tissue collected for the Biobank has already provided a large volume of data for the clinicians and scientists working within the Medical Research Council Centre for Reproductive Health at the University of Edinburgh, and will no doubt continue to develop as a resource for both the University of Edinburgh and its collaborators for many years to come.

All materials and reagents used are detailed in Appendix 1.

Recipes for reagents are provided in Appendix 2.

2.2. SAMPLE COLLECTION

2.2.1. Ethical approval and consent

Approval from the Lothian Local Research Ethics Committee was granted for all experimental work described in this thesis. Written information was provided and informed, written consent was obtained from participants who donated blood or tissue samples.

2.2.2. Sample collection

2.2.2.1. Samples used in quantitative RT-PCR and Immunohistochemistry experiments

For the quantitative real time polymerase chain reaction (quantitative RT-PCR) experiments described in this thesis which compare relative mRNA expression in labouring and non-labouring term women, tissue samples were collected from a total of 22 women, 11 labouring and 11 non-labouring. Samples used in immunohistochemistry analysis of labouring and non-labouring tissues were also taken from these cohorts of patients. The tissue samples used were myometrium, placenta, amnion and chorio-decidua. Samples were collected and stored as described in the sections above until ready for use. Patients were recruited according to the following inclusion and exclusion criteria and a table of patient demographics follows (Table 2.1). There was no difference in age, BMI or gestation between the two groups (t-tests, $p>0.05$) but non-labouring women were more likely to be parous than labouring women.

Term Pregnant Non-Labouring Women

Healthy pregnant women at term presenting to hospital for elective caesarean section were recruited. Term was defined as 37 completed weeks of pregnancy or greater, based on the Estimated Due Date (EDD) calculated at booking. EDD was calculated from the ultrasound scan (USS) performed before 16 weeks gestation. In women who booked after 16 weeks gestation, gestational age was calculated from the last menstrual period (LMP) correlated with measurements from their first USS. Women

were excluded if there was discrepancy in dates of greater than seven days or other uncertainty about gestational age. Indications for elective caesarean section included breech presentation, previous caesarean section, placenta praevia and maternal request. Exclusion criteria included hypertensive disorders of pregnancy, diabetes mellitus and gestational diabetes, multiple pregnancy and the use of regular steroid medication including inhaled steroids for asthma.

Term Pregnant Labouring Women

Healthy pregnant women in labour at term were recruited. Term was defined as 37 completed weeks of pregnancy or greater, as for non-labouring women. Labour was defined as regular contractions and dilation of the cervix of greater than or equal to 3cm. The indications for women undergoing emergency caesarean section included breech presentation, fetal distress and failure to progress in labour or second stage, with a cervical dilatation of between 3cm and 10cm. Exclusion criteria included hypertensive disorders of pregnancy, diabetes mellitus and gestational diabetes, multiple pregnancy, signs of clinical infection and the use of regular steroid medication including inhaled steroids for asthma. Women who were induced with vaginal prostaglandins were also excluded. The use of syntocinon infusion was not an exclusion criterion in this group.

Group	**Age (years) Mean ± SEM**	**BMI (kg/m^2) Mean ± SEM**	**Gestation (days) Mean ± SEM**	**Parity (median)**
Labouring (n=11)	29.73 ± 1.49	24.56 ± 1.61	283 ± 2.1	P0
Non-labouring (n=11)	32.91 ± 1.81	23.2 ± 0.36	278 ± 1.9	P1

Table 2.1 Labouring and Non-Labouring Demographics

Demographics of 22 patients from whom tissue was collected at caesarean section. There was no difference in age, BMI or gestation between the two groups (t-tests,

p>0.05) but non-labouring women were more likely to be parous than labouring women.

2.2.2.2. Tissue samples used in tissue culture experiments

Where myometrial samples were required for tissue culture experiments, the samples were placed into Roswell Park Memorial Institute (RPMI) 1640 culture medium and transported immediately back to the laboratory and stored at 4°C until used that same day. Where such samples were used, the cohorts of patients and experimental protocols are described in each relevant chapter.

2.2.2.3. Peripheral venous blood

In the case of women undergoing elective caesarean section, venous maternal blood samples were drawn from the forearm via a venous catheter at the time of siting. The venous catheter was sited just before siting of the spinal anaesthetic at the beginning of the operation. In the case of women in labour, blood samples were either drawn from the forearm via a venous catheter at the time of insertion, or from an antecubital vein using a 21-gauge needle. In the case of non-pregnant women or women at earlier gestation than term, blood samples were drawn from an antecubital vein using a 21-gauge needle. Samples were drawn into Sarstedt Monovette serum gel blood-collection tubes for isolation of serum. Blood samples were put onto ice and transported back to the lab where they were centrifuged at 2000 revolutions per minute (rpm) for ten minutes. The serum layers were drawn off and stored at -80°C until needed. A further description of the patient cohorts from which samples were obtained is to be found in the Methods section of the relevant chapter.

2.3. ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISAs are used to quantify the amount of a particular protein in a solution, by comparing measured levels in a sample with a standard curve derived from samples containing known concentrations of the protein.

The assay performed in this work was a competitive ELISA. A competitive ELISA operates on the basis of competition between the enzyme conjugate and the protein of interest in the sample for a limited number of antibody binding sites. A plate is used which has wells coated with antibodies raised against the protein which is to be measured. The solution of interest, or the standard is then added to the plate, followed by the enzyme conjugate. During incubation, binding takes place in which both the enzyme conjugate and the protein of interest are competing for binding with the antibody adherent to the well. After incubation, the plate is washed which removes all of the unbound material. Next a substrate is added which generates a colour where binding of the enzyme conjugate is detected. Quantitative results are obtained by measuring and comparing the absorbance reading of the sample wells against the standard wells using spectrophotometry.

2.3.1. LXA4 ELISA

A commercially available LXA4 assay kit was used, containing plates pre-coated with capture antibody, and all required reagents and buffers. Samples and standards (50µl, range 0.02 to 2 ng/ml) were added to the appropriate wells in duplicate. Concentrated LXA4 enzyme conjugate was diluted with the supplied EIA buffer, applied to each well (50µl) and incubated at room temperature for one hour on an orbital plate shaker. After incubation, the contents of the plate were discarded and the wells washed with the provided wash buffer for three washings. Substrate was added to each well (150µl) and the plate was gently shaken and then incubated for 30 minutes at room temperature. The plate was read in a microplate reader at 650nm optical density.

2.4. IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESENCE

Immunohistochemistry is a means by which localisation of an antigen within a section of tissue is determined by a specific primary antibody. A variety of detection systems may then be used to detect and visualise the specific staining.

Immunohistochemistry experiments described in this thesis were performed on tissue samples of myometrium, placenta and fetal membranes in the form of rolls of amnion and chorio-decidua. Collection, fixation of the sample and storage in paraffin blocks is described previously.

Sections 5μm thick were cut from the paraffin embedded tissues and mounted on slides, deparaffinised in xylene and rehydrated in a graded alcohol series. Specific protocols are now described.

2.4.1. Specific protocols

2.4.1.1. FPR2/ALX

Antigen retrieval was performed by pressure cooking for 5 minutes in 10mM citrate buffer pH 6 and then cooled for 20 minutes at room temperature. Endogenous peroxidase was blocked using a 3% hydrogen peroxide in methanol solution for 30 minutes followed by washing in water (5 minutes) and phosphate buffered saline (PBS) (5 minutes). Sections were blocked in normal goat serum diluted 1:5 with PBS and 5% bovine serum albumin (NGS/PBS/BSA) for 30 minutes, followed by Avidin/Biotin blocking using a kit according to manufacturers instructions and washing in between with PBS (5 minutes x 2). Incubation with the primary antibody was performed overnight at 4°C. The primary antibody used was rabbit polyclonal anti-FPR2/ALX antibody at 1:200 (diluted in NGS/PBS/BSA). After washing in PBS (5 minutes x 2) the sections were incubated with goat anti-rabbit biotinylated secondary antibody diluted 1:500 in blocking serum for 30 minutes and washed again in PBS (5 minutes x 2). The sections were incubated with Streptavadin–HRP

diluted 1:1000 in PBS for 30 minutes and washed again in PBS (5 minutes x 2), followed by 3,3-diaminobenzidine (DAB) detection (DAB diluted at 1 drop/ml). Colour development was monitored microscopically and stopped by washing in water. Sections were counterstained in haematoxylin, Scotts tap water used to provide blue nuclear staining, dehydrated and coverslipped using pertex.

Two negative controls were used, the first with the primary antibody omitted and the second using a rabbit monoclonal IgG1 at the same concentration as the primary antibody.

2.4.1.2. ANXA1

An identical protocol was used to the protocol described for FPR2/ALX, other than differing primary and secondary antibodies were used. The primary antibody used was mouse anti-AnnexinA1 at 1: 200 (diluted in NGS/TBS/BSA). Since the primary antibody was raised in mouse, the secondary antibody used was goat anti-mouse biotinylated secondary antibody diluted 1:500 in the blocking serum.

Two negative controls were used, the first with the primary antibody omitted and the second using a mouse monoclonal IgG1 at the same concentration as the primary antibody.

2.4.1.3. Immunofluoresence to co-localise FPR2/ALX and neutrophil elastase

Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide in methanol for 30 minutes. Sections were then blocked in normal goat serum diluted 1:5 with PBS and 5% bovine serum albumin (NGS/PBS/BSA) for 30 minutes and then incubated with mouse anti-neutrophil elastase (1:500). The following day slides were washed in PBS (5 minutes x 2), incubated with the secondary antibody, goat anti-mouse Fab at a concentration of 1:500 in NGS/PBS/BSA for 30 minutes and washed again in PBS (5 minutes x 2). The sections were incubated for 10 minutes with tyramide red signal amplification kit, diluted 1:50 in the supplied buffer, to amplify the staining with red fluorescence, then washed in PBS (5 minutes x 2). Sections were then pressure-cooked for 5 minutes in

10mM citrate buffer, pH 6), cooled for 20 minutes at room temperature and then blocked with NGS/PBS/BSA for 30 minutes. The sections were then incubated with rabbit polyclonal anti-FPR2/ALX primary antibody (1:1000) overnight at 4°C. The following day, sections were washed in PBS (5 minutes x 2) and incubated with goat anti-rabbit Fab secondary antibody (1:500) for 30 minutes, washed again in PBS (5 minutes x 2) and then were incubated for 10 minutes with tyramide green signal amplification kit, diluted 1:50 in the supplied buffer, to amplify the staining with green fluorescence. Sections were washed finally with PBS (5 minutes x 2) and incubated with nuclear counterstain DAPI for 10 minutes (1:1000), mounted in Permafluor, coverslipped, and visualised using a laser-scanning microscope (LSM 710; Carl Zeiss, Jena, Germany) using a x40 1.4 numerical aperture oil-immersion lens. Sections were scanned with 405 (nuclear staining), 488 (FPR2/ALX) and 543 (smooth muscle elastase) laser lines, and images were acquired using Zen 2009 5.5 software (Carl Zeiss).

2.5. TAQMAN QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (RT-PCR)

2.5.1. RNA extraction

RNA was extracted from the samples using a combined TRIzol and RNeasy columns protocol. The RNeasy system is a commercially available kit which uses centrifuge columns containing silica-gel membranes to selectively bind RNA. Salt and ethanol containing buffers are applied to wash away contaminants and the RNA eluted with water. An alternative method for extracting RNA involves using TRIzol and other reagents to separate samples into layers from which RNA can be isolated. This combined protocol was developed by colleagues in our laboratory and was found to produce samples of RNA of both good quality and concentration, with low contamination.

Samples were thawed and removed to secure-lock Eppendorf tubes containing TRIzol (0.5ml for tissue less than 50mg, 1 ml for tissue between 50 and 100mg) and a sterile ball bearing. Samples were homogenized at 25Hz for 4 minutes, with the plates changed over at 2 minutes. The homogenized samples were then allowed to incubate for 15 minutes at room temperature to allow dissociation of nucleoprotein complexes. The samples were centrifuged at 14000rpm for 10 minutes and the supernatant transferred to a phase-lock tube. 100µl of chloroform per 0.5ml TRIzol was added, the tubes shaken vigorously for 15 seconds and then centrifuged at 12000g for 15 minutes at 4°C. The aqueous phase was removed to a fresh tube, one volume of 70% ethanol added and mixed by pipetting and then this solution applied to the RNeasy column. RNA extraction was then completed as per the kit instructions as follows. Columns were centrifuged for 15 seconds at 10000rpm and the eluate discarded. On-column DNase digestion was then performed using the kit manufacturer's instructions. This involves applying 80µl of DNase1 solution onto each column and incubating at room temperature for 15 minutes. Following this 700µl of Buffer RW1 was applied to each column and then the columns centrifuged at 10000rpm for 15 seconds. Eluate was discarded and 500 µl of Buffer RPE applied to each column and then the columns centrifuged at 10000rpm for 15 seconds. This

step was repeated but with a 2 minute centrifugation. The columns were then transferred to fresh tubes and 30µl DNase-free, RN-ase free sterile water applied to the membrane. The columns were incubated for 1 minute at room temperature and then centrifuged at 10000rpm for 1 minute. The eluates were reapplied to the membranes and re-centrifuged. Columns were subsequently discarded, with the eluate containing the extracted RNA.

2.5.2. Estimation of RNA concentration and integrity

To estimate the concentration of RNA extracted from tissue samples, spectophotometry was performed using a Nanodrop 1000 spectophotometer (ThermoScientific, Wilmington, DE, USA). Absorbancy at 260nm and 280nm was measured and the concentrations given in ng/µl. A 260:280 value of around 1.8 indicates a pure RNA sample. Ratios of 1.7 to 2.1 were taken to be acceptable. RNA was stored at -80° for further use.

2.5.3. cDNA synthesis

200ng of total RNA of each sample was reverse transcribed into cDNA using the GeneAmp RNA PCR kit under manufacturer's instructions. This involved making up a mixture of Reverse Transcriptase buffer, Reverse Transcriptase, deoxynucleotide triphosphates (dNTPs), RNase inhibitor (all included in the kit) and RNA sample according to manufacturer's instructions. Transcription was performed using a G-Storm GS1 thermal cycler, with a cycle of 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 minutes. Samples were stored at -20°C until needed.

2.5.4. Quantitative RT-PCR

TaqMan quantitative RT-PCR was used to determine relative gene expression. The probe for the gene of interest was labelled with the reporter dye FAM and the 18S probe labelled with the reporter dye VIC at the 5'end of the oligonucleotide. A quencher dye (TAMRA) was used to label the 3' ends of both probes. Expression of the analysed genes was normalised using an 18S ribosomal RNA (rRNA) reference gene. The amount of ribosomal 18S in samples is constant, relative to the amount of cDNA present, thus quantification of the gene of interest can be related to the

abundance of ribosomal 18S. This value was then related to an internal control sample.

A reaction mix was made containing PCR reaction Mastermix, 18S control primers and probes, forward and reverse primers and hybridisation probe specific for the gene of interest. Samples were run in duplicate, on TaqMan Fast optical PCR plates, with each replicate containing reaction mix and cDNA. The exact composition of the reaction mix did vary between experiments depending on the gene of interest and so recipes for the reaction mix used and further details of the specific primers and probes are given where appropriate in the Methods sections of the relevant chapters. Three negative controls were included on each plate as follows. An RT-negative sample was used to exclude genomic DNA contamination. This sample was produced at the time of cDNA manufacture by excluding the reverse transcriptase enzyme from a sample containing RNA template. The second control was an RT-water sample used to ensure no contamination in the cDNA manufacturing process and was produced by replacing the RNA template with water. The third control was a Taqman-reaction negative control which replaced cDNA with water and controlled for contamination of the reaction mix. Wells were sealed with an optical adhesive film, and the reaction run on the ABI Prism 7500 Sequence Detection System (Applied Biosystems, UK). PCR conditions were as follows: 50°C – 2min, 95°C – 10min, (95°C – 15secs, 60°C – 1min) x 40 for both genes.

2.5.5. Data analysis

Data analysis was carried out using SDS Version 2.1 program (Applied Biosystems). The baseline value was set 2 or 3 cycles before the earliest amplification and the threshold value was set in the linear phase of the exponential region of the amplification plot. The comparative cycle threshold (Ct) method was used to determine relative gene expression. Ct is the cycle number at which the PCR signal crosses the set threshold. The average Ct values for replicate wells was determined. ΔCt was then calculated by subtracting the 18S Ct from the target gene Ct. $\Delta\Delta$Ct was calculated by subtracting the ΔCt of the control sample from the ΔCt for each sample. The fold change in target gene expression was compared to the reference

sample using the formula 2^(-ΔΔCt), thus giving a relative value to the control sample.

3. The role of lipoxin A4 in the human pregnant reproductive tissues

3.1. INTRODUCTION

Human labour is an inflammatory event. At or just after the onset of labour, there is increased tissue expression of pro-inflammatory cytokines (Osman et al. 2003), and an influx of leucocytes into the myometrium, cervix and fetal membranes (Thomson et al. 1999; Osman et al. 2003), which provide a further source of pro-inflammatory cytokines (Young et al. 2002). These cytokines subsequently attract more leucocytes (Elliott et al. 2000), are involved in tissue remodelling at the cervix (Sennstrom et al. 2000) and in the fetal membranes and have stimulatory effects on myometrial contractility (Tribe et al. 2003).

It is now recognised that a normal inflammatory response involves pro-inflammatory processes responding to and neutralising the initiating insult but also, running in parallel to this, anti-inflammatory and pro-resolution mechanisms. These mechanisms regulate and limit the pro-inflammatory response and ensure that it is brought to a timely end in order to limit damage to host tissue (Serhan and Savill 2005; Serhan et al. 2007).

The lipoxins are endogenous lipid mediators which have been demonstrated in other systems in the human body to have anti-inflammatory and pro-resolution effects. The anti-inflammatory and pro-resolution properties of LXA4 include inhibition of neutrophilic chemotaxis, adherence to vascular endothelial cells and trafficking across epithelial cell monolayers (Serhan et al. 1995; Papayianni et al. 1996; Filep et al. 1999). It also promotes the phagocytosis of apoptotic neutrophils and clearance from the site of inflammation (Godson et al. 2000; Mitchell et al. 2002). LXA4 is generated from arachidonic acid, via the actions of the lipoxygenase enzymes, 5-, 12- and 15-lipoxygenase (ALOX5, ALOX12 and ALOX15). The pathways involved in the production of LXA4 are described in more detail in section 1.4.1.1, but essentially the production is via ALOX15 and ALOX5 at mucosal surfaces (Samuelsson et al. 1987), or via ALOX5 and ALOX12 in a platelet-neutrophil reaction (Serhan and Sheppard 1990). The receptor by which LXA4 signals has been identified as formyl peptide receptor 2 (FPR2/ALX), a G-protein coupled receptor

(Maddox et al. 1997). FPR2/ALX is a promiscuous receptor, through which a number of different ligands signal producing both pro- and anti-inflammatory effects (Ye et al. 2009).

Much data have been published supporting the concept that labour is an inflammatory process. However, the vast majority of these data focuses on pro-inflammatory processes, with relatively little described in regard to anti-inflammatory or pro-resolution mechanisms. It may be that pro-resolution mediators such as LXA4 have an important role to play in the delicate balance between pregnancy and labour, and possibly in the initiation of labour itself.

3.1.1. Aims

This chapter aims to:

- explore the expression of the enzymes involved in the synthesis of LXA4 and its receptor in labouring and non-labouring reproductive tissues
- determine the localisation of FPR2/ALX by immunohistochemistry
- and examine the production of LXA4 in non-pregnant, pregnant and labouring women.

3.2. METHODS

3.2.1. Recruitment of patients

3.2.1.1. Serum samples for use in ELISA

Women were recruited from four cohorts (detailed in Table 3.1) and written informed consent to collect blood samples was obtained. All participants were healthy and pregnant participants had uncomplicated, singleton pregnancies.

	Characteristics of group	**Number of samples taken from each participant**	**Number of participants in cohort**
Cohort 1	Non-pregnant, premenopausal women	1 sample	9
Cohort 2	Pregnant women	3 samples 24 weeks gestation 31 weeks gestation 37 weeks gestation	8
Cohort 3	Pregnant women at term, prior to the onset of labour, gestation 39+4 ± 1.9 (mean ± SEM)	1 sample	10
Cohort 4	Pregnant women at term, during the first stage of labour, gestation 40+2 ± 2.7 (mean ± SEM)	1 sample	10

Table 3.1 Cohorts of participants from whom blood samples were obtained

3.2.1.2. Tissue samples for use in RT-PCR and Immunohistochemistry experiments

Groups of labouring (n=11) and non-labouring women (n=11) were recruited and informed, written consent was obtained to provide tissue samples as described in section 2.2.1. Inclusion and exclusion criteria for these groups are described in section 2.2.2.1.

3.2.2. Sample collection

3.2.2.1. Serum samples for use in ELISA

Peripheral venous blood samples were drawn from an antecubital vein into a Sarstedt Monovette Serum Gel tube, chilled on ice and transported to the laboratory. Serum was separated by centrifugation (2000 rpm for 10 minutes) and samples stored at -80°C until further analysis.

3.2.2.2. Tissue samples for use in RT-PCR and Immunohistochemistry experiments

Samples of myometrium, placenta, amnion and chorio-decidua were taken at the time of elective or emergency caesarean section, as described in section 2.1.5.1.

3.2.3. ELISA for lipoxin A4

Levels of LXA4 were measured in the serum samples by ELISA, as described in section 2.3.1. This ELISA was performed by Dr David Maldonado-Perez, whose assistance is gratefully acknowledged.

3.2.3.1. Data analysis

Longitudinal samples taken at intervals during pregnancy were compared to each other using repeated-measures analysis of variance (ANOVA) and Tukey's multiple comparison post test. A repeated-measures ANOVA was used because the samples are matched. When comparing other groups the samples are not matched as they are different cohorts of women as opposed to repeated samples taken at intervals from the same women. Therefore a one-way analysis of variance with Tukey's multiple comparison post test was used. The comparison between labouring and non-labourng term samples was performed using an unpaired t-test. All statistical calculations were carried out using GraphPad Prism 5.02 (GraphPad software, CA, USA).

3.2.4. Immunohistochemistry of FPR2/ALX

Immunohistochemistry was performed on samples of labouring and non-labouring myometrium, placenta and fetal membranes to localise FPR2/ALX and immunofluoresent co-localisation with neutrophils in labouring and non-labouring myometrium was performed.

3.2.4.1. Immunohistochemistry

The protocol used for determining the localisation of FPR2/ALX by immunohistochemistry is described in section 2.4.1.1 and was performed on sections cut from myometrial, placental and membrane biopsies, taken either at elective or emergency caesarean section.

3.2.4.2. Immunofluoresence to co-localise FPR2/ALX and neutrophil elastase

The protocol used for co-localising FPR2/ALX and neutrophil elastase by immunohistochemistry is described in section 2.4.1.3 and was performed on sections cut from myometrial, placental and membrane biopsies, taken either at elective or emergency caesarean section.

3.2.5. Quantitative RT-PCR

Quantitative RT-PCR was performed to determine relative mRNA expression of ALOX5, ALOX12, ALOX15 and FPR2/ALX in labouring and non-labouring myometrium, placenta, amnion and chorio-decidua.

Total RNA was extracted from the biopsies of myometrium, placenta, amnion and chorio-decidua, RNA concentrations were estimated and cDNA was synthesised, as described in section 2.5. Quantitative RT-PCR was performed as described in section 2.5. Specific reaction mix composition and primers and probes used are detailed below.

Primers were designed by Dr David Maldonado-Perez, using the ProbeFinder version 2.42 for Human (Roche Applied Science) and the corresponding probes from the Universal Probe Library (UPL) Set (Roche Applied Science) were used to identify

and quantify cDNA. Sequences and probes for the measured genes are described in Table 3.2.

The reaction mix used for determination of relative gene expression was the same for ALOX5 and FPR2/ALX, although the primers and probes differed for each gene. PCR was performed with a final volume of 25µl per well, containing 12.5µl 2x Express Mastermix, 0.25µl of forward primer, 0.25µl reverse primer, 0.25µl probe, 1.25µl 18S primer/probe assay mix, 1µl cDNA and 8.65µl RNase-free, DNase-free sterile water.

For the genes ALOX 12 and ALOX15, an alternative experimental protocol was followed, using a different 'Mastermix' which had been demonstrated in our laboratory to have higher sensitivity in picking up low levels of gene expression (personal communication, Sharon Battersby, Lab Technician, University of Edinburgh). PCR was performed with a final volume of 25ul per well, containing 12.5ul 2x Express Mastermix, 0.25µl of forward primer, 0.25µl reverse primer, 0.25µl probe, 0.375 18S primer/probe assay mix, 1ul cDNA and 20.375ul RNase-free, DNase-free sterile water.

Gene	Forward Primer	Reverse Primer	Probe Number
ALOX5	5'-GGAAACACGGCAAAAACAAT	5'-ATCGATGCTCAAGGGGAAG	58
ALOX12	5'-CTGAAGATGGAGCCCAATG	5'-ACAGTGTTGGGGTTGGAGAG	75
ALOX15	5'-TCTTGCCCATGGTCATCC	5'-AGGCAAGAAAAGGGGAGGT	78
FPR2	5'-GCACACAGGAAAAGGAGCTTA	5'-AGCCAGCAGACTCATAGGACA	82

Table 3.2 Sequences and UPL probe numbers used

3.2.5.1. Data analysis

A Mann-Whitney test was performed to compare relative gene expression in samples of labouring and non-labouring myometrium, placenta, amnion and chorio-decidua. To compare the relative levels of mRNA expression between the four different types of tissue in the labouring group a Friedman test with Dunns Multiple Comparison post test was performed. The Friedman test assumes non-parametric data, and that values in each row represent matched data. This test was chosen in view of the fact that the four different types of tissue (ie. myometrium, placenta, amnion and chorio-decidua) were taken from the same eleven women and could therefore be considered as matched data. The same tests were performed for the data from the non-labouring group. All statistical calculations were carried out using GraphPad Prism 5.02 (GraphPad software, CA, USA).

3.3. RESULTS

3.3.1. Circulating lipoxin A4 levels were greater in pregnant women than in non-pregnant women

LXA4 was detected by ELISA in the peripheral blood of both non-pregnant and pregnant women, in all the studied samples. Mean levels were higher in pregnant women at all measured gestations than in non-pregnant women ($p<0.0001$, Figure 3.1), and at 37 weeks were 14.9-fold greater than the mean non-pregnant levels. As pregnancy progressed from 24 to 37 weeks of gestation, circulating levels of LXA4 rose, with a 1.5-fold increase between 37 weeks and 24 weeks, which was a significant difference ($p=0.04$, Figure 3.1).

Levels of circulating LXA4 were no different in women who were in labour from women who were not in labour and undergoing elective caesarean delivery ($p=0.99$, Figure 3.2). The women in these two cohorts were gestation matched to each other and had a mean gestation of 40 weeks ± 1.7 days. When these two cohorts were compared to the samples from the women at 37 weeks gestation, the levels were found to be significantly higher in the 37 week cohort, with a two-fold difference in the levels ($p<0.0001$). This suggests that LXA4 levels increase towards term but actually fall as delivery itself approaches.

Together these data demonstrate an increase in circulating LXA4 levels during pregnancy, with an increase throughout the second trimester until term, at which point a peak level was observed. Levels appear to fall in the final few weeks of pregnancy, but there was no further change at the onset of labour.

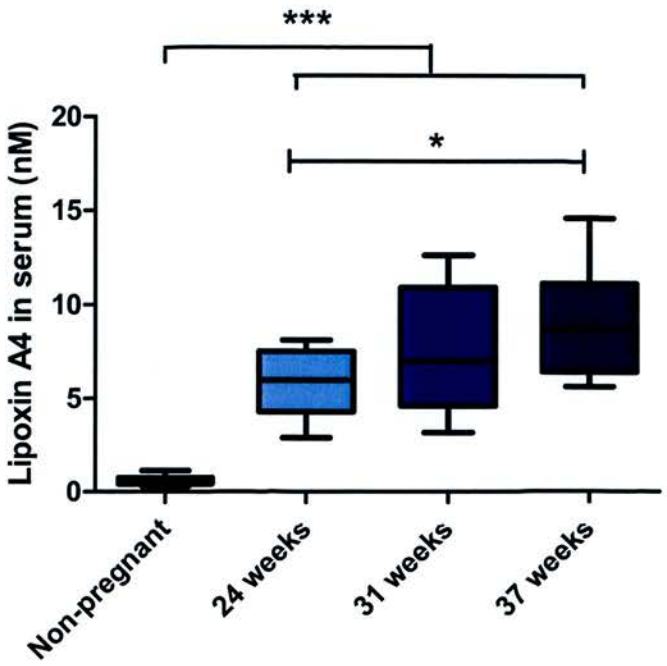


Figure 3.1
Circulating LXA4 levels in pregnant and non-pregnant women

Circulating lipoxin A4 levels are significantly greater in pregnant women at 24, 31 and 37 weeks gestation when compared to non-pregnant women, and increase between 24 and 37 weeks gestation. This figure illustrates levels of LXA4 in serum of non-pregnant women (n=9) and a cohort of pregnant women from whom serum samples were taken at 24, 32 and 37 weeks gestation (n=8). Longitudinal pregnant samples were analysed with Repeated Measures ANOVA and Tukey post test, pregnant vs non-pregnant analyses were performed using one way analysis of variance and Tukey post test. *P <0.05; ***P <0.001.

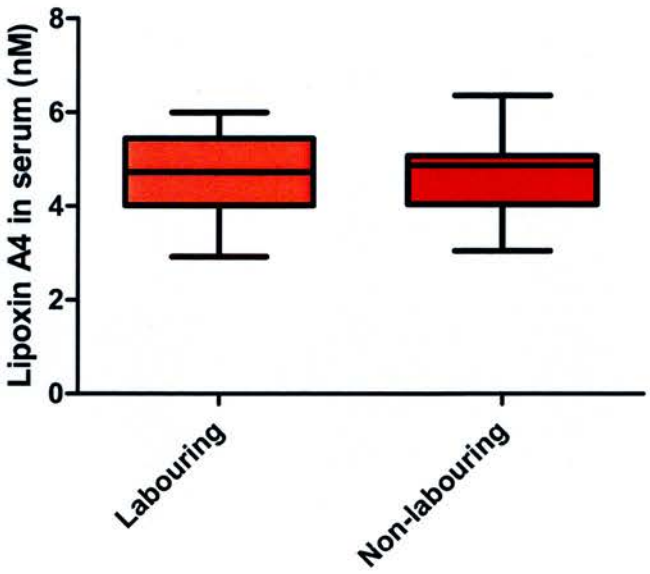


Figure 3.2
Circulating LXA4 levels at term

Circulating levels of lipoxin A4 are not significantly different in term women in labour compared to term women not in labour. This figure illustrates levels of LXA4 in serum of pregnant women at term who were either in labour or not labouring (n=10 in each group). Unpaired t test, p=0.99.

3.3.2. mRNA expression of ALOX5 was greater in labouring myometrium and chorio-decidua than in non-labouring.

Expression of ALOX15 was not detected in any of the tissue samples, either labouring or non-labouring. Expression of ALOX12 was detected in some, but not all samples studied, and was present at low levels. It was not possible to reliably compare relative expression levels of this gene between the labouring and non-labouring groups due to the low levels of expression.

In contrast, mRNA expression of ALOX5 was detected in all four of the studied tissues (Figures 3.3-3.6). In myometrium, relative mRNA expression was greater in labouring tissue when compared to non-labouring, (median fold change 2.6, $p=0.0025$, Fig 3.3). Relative expression of ALOX5 was also greater in labouring chorio-decidua when compared to non-labouring, (median fold change of 4.8, $p=0.0058$, Fig 3.6). There was no difference in expression between labouring and non-labouring placenta (Fig 3.4), nor between labouring and non-labouring amnion (Fig 3.5).

When relative expression levels were compared between the four non-labouring tissue types, it was observed that greater expression was found in placenta than in either amnion or chorio-decidua (Fig 3.7). However, when labouring tissues were compared, there was no difference in relative expression between any of the tissues, presumably reflecting the change in expression levels occurring with labour (Fig 3.8).

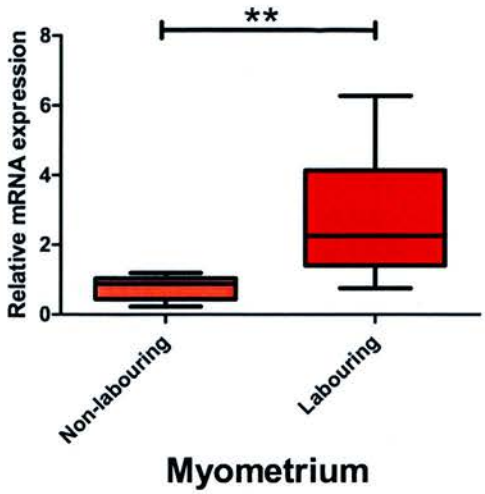


Figure 3.3 ALOX5 mRNA expression in myometrium
Relative mRNA expression of ALOX5 is significantly greater in labouring myometrium compared to non-labouring (median fold change 2.6). Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.0025, Mann-Whitney test.

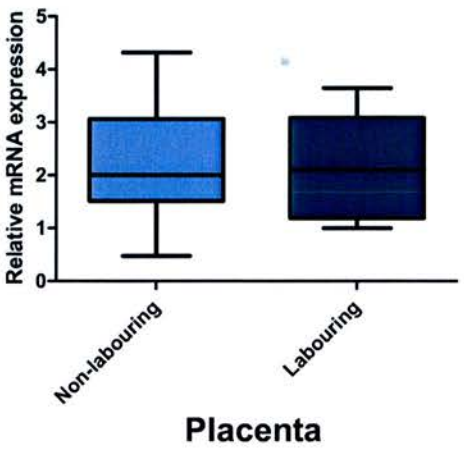


Figure 3.4 ALOX5 mRNA expression in placenta
Relative mRNA expression of ALOX5 is not significantly different in labouring placenta compared to non-labouring. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.95, Mann-Whitney test.

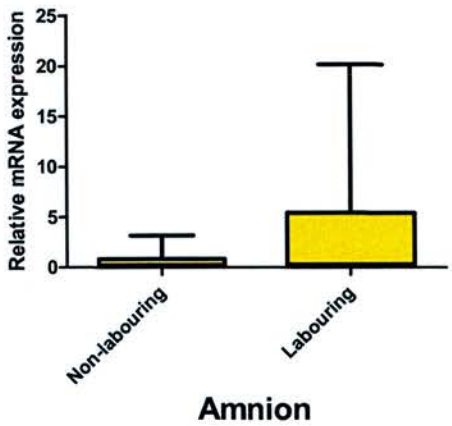


Figure 3.5 ALOX5 mRNA expression in amnion
Relative mRNA expression of ALOX5 is not significantly different in labouring amnion compared to non-labouring. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.21, Mann-Whitney test.

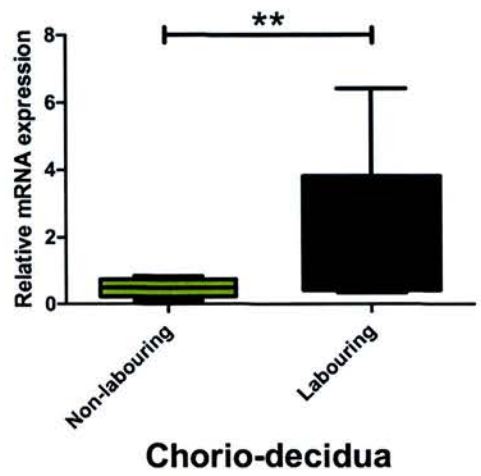


Figure 3.6 ALOX5 mRNA expression in chorio-decidua
Relative mRNA expression of ALOX5 is significantly greater in labouring chorio-decidua compared to non-labouring (median fold change 4.8). Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.0058, Mann-Whitney test.

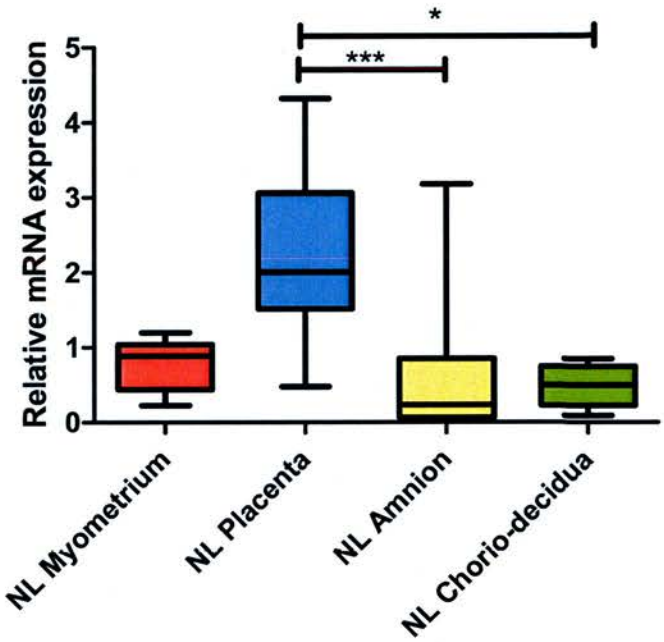


Figure 3.7 ALOX5 mRNA expression in non-labouring tissues
Relative mRNA expression of ALOX5 is greater in placenta than in amnion or chorio-decidua, but no other statistically significant differences are observed. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11; p=0.0008; Friedman test with Dunns post test. ***p<0.001, *p<0.05

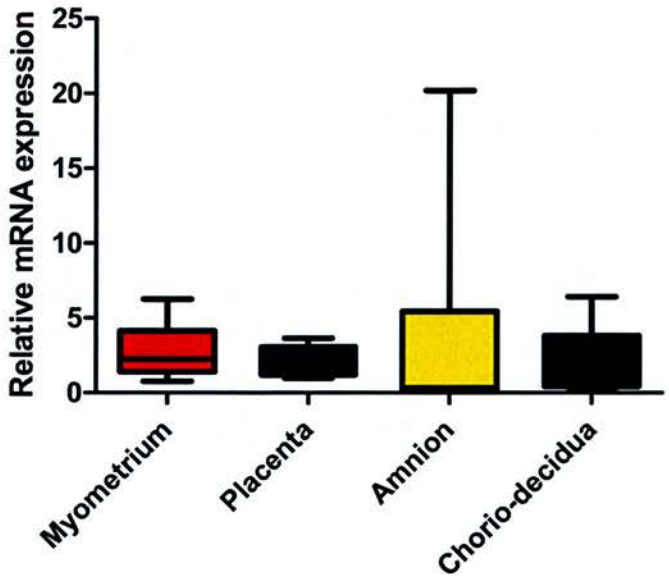


Figure 3.8 ALOX5 mRNA expression in labouring tissues
Relative mRNA expression of ALOX5 is not significantly different in the four labouring tissues of myometrium, placenta, amnion and chorio-decidua. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11; p=0.09; Friedman test with Dunns post test.

3.3.3. mRNA expression of FPR2/ALX was greater in labouring myometrium, placenta, amnion and chorio-decidua than in non-labouring.

FPR2/ALX mRNA was detected in term myometrium, placenta, amnion, and chorio-decidua from both labouring and non-labouring women (Figs 3.9-3.12). Relative mRNA expression was greater in labouring myometrium (median fold change 7.2, p=0.0016, Fig 3.9), placenta (median fold change 2.1, p=0.049, Fig 3.10), amnion (median fold change 6.9, p=0.018, Fig 3.11) and chorio-decidua (median fold change 96, p=0.0016, Fig 3.12).

In the non-labouring women relative mRNA expression of FPR2/ALX was greater in placenta than in chorio-decidua (mean fold change 2.3) but there were no other relative differences in expression between the tissues (Fig 3.13). In labouring tissues there were no significant differences in relative mRNA expression of FPR2/ALX between the four tissues (Fig 3.14).

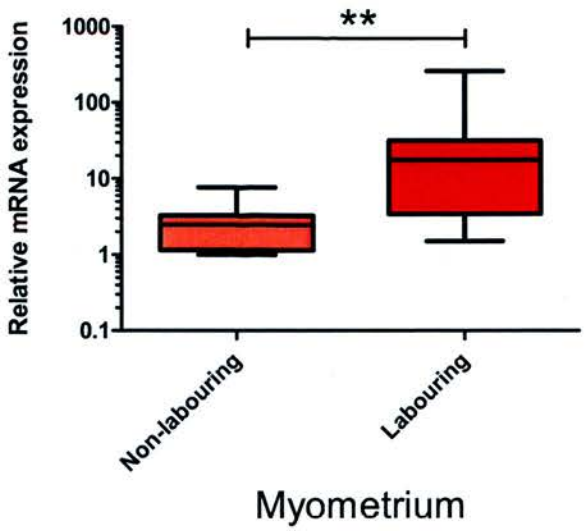


Figure 3.9 FPR2/ALX mRNA expression in myometrium
Relative mRNA expression of FPR2/ALX is significantly greater in labouring myometrium compared to non-labouring (median fold change 7.2). Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.0016, Mann-Whitney test.

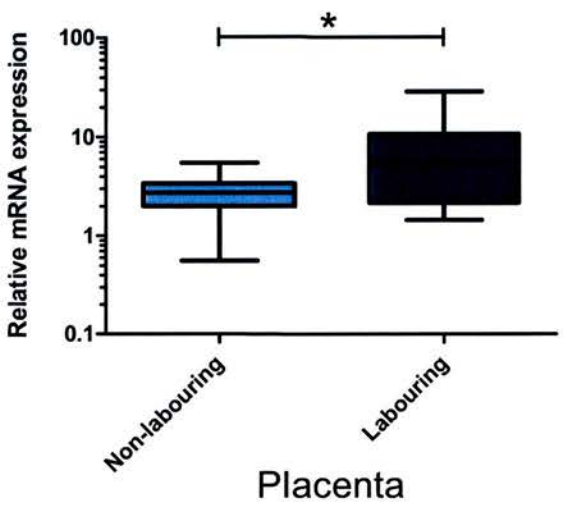


Figure 3.10 FPR2/ALX mRNA expression in placenta
Relative mRNA expression of FPR2/ALX is significantly greater in labouring placenta compared to non-labouring (median fold change 2.1). Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.049, Mann-Whitney test.

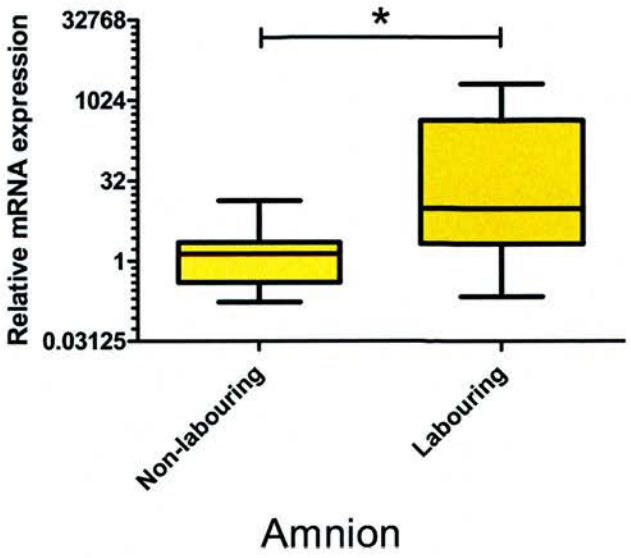


Figure 3.11 FPR2/ALX mRNA expression in amnion
Relative mRNA expression of FPR2/ALX is significantly greater in labouring amnion compared to non-labouring (median fold change 6.9). Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.018, Mann-Whitney test.

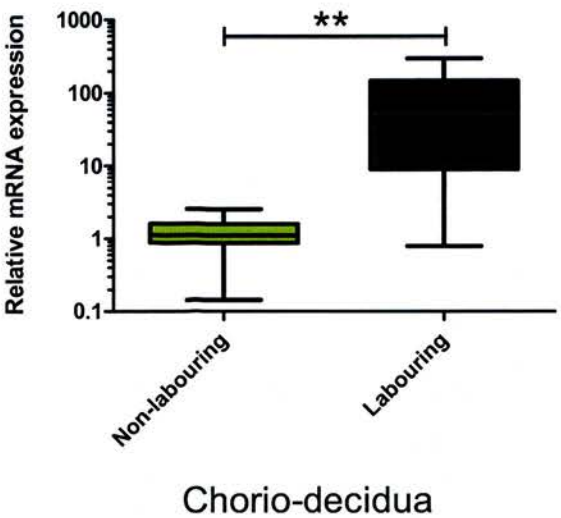


Figure 3.12 FPR2/ALX mRNA expression in chorio-decidua
Relative mRNA expression of FPR2/ALX is significantly greater in labouring chorio-decidua compared to non-labouring (median fold change 96). Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.0016, Mann-Whitney test.

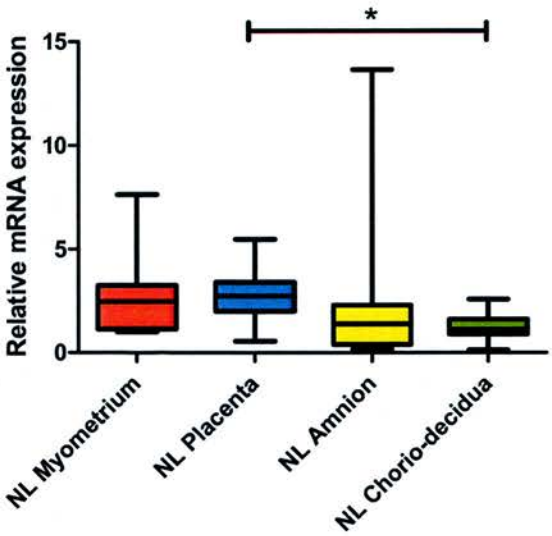


Figure 3.13 FPR2/ALX mRNA expression in non-labouring tissues
Relative mRNA expression of FPR2/ALX is significantly greater in non-labouring (NL) placenta than in NL chorio-decidua (median fold change 2.3), but no other statistically significant differences are observed. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11; p=0.048; Friedman test with Dunns post test, *p<0.05

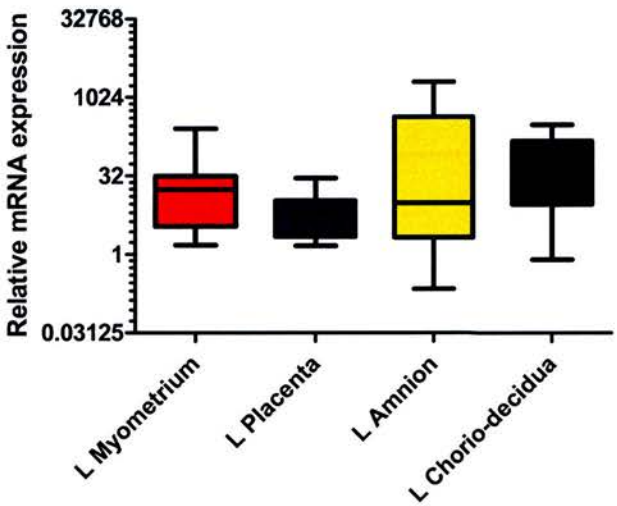


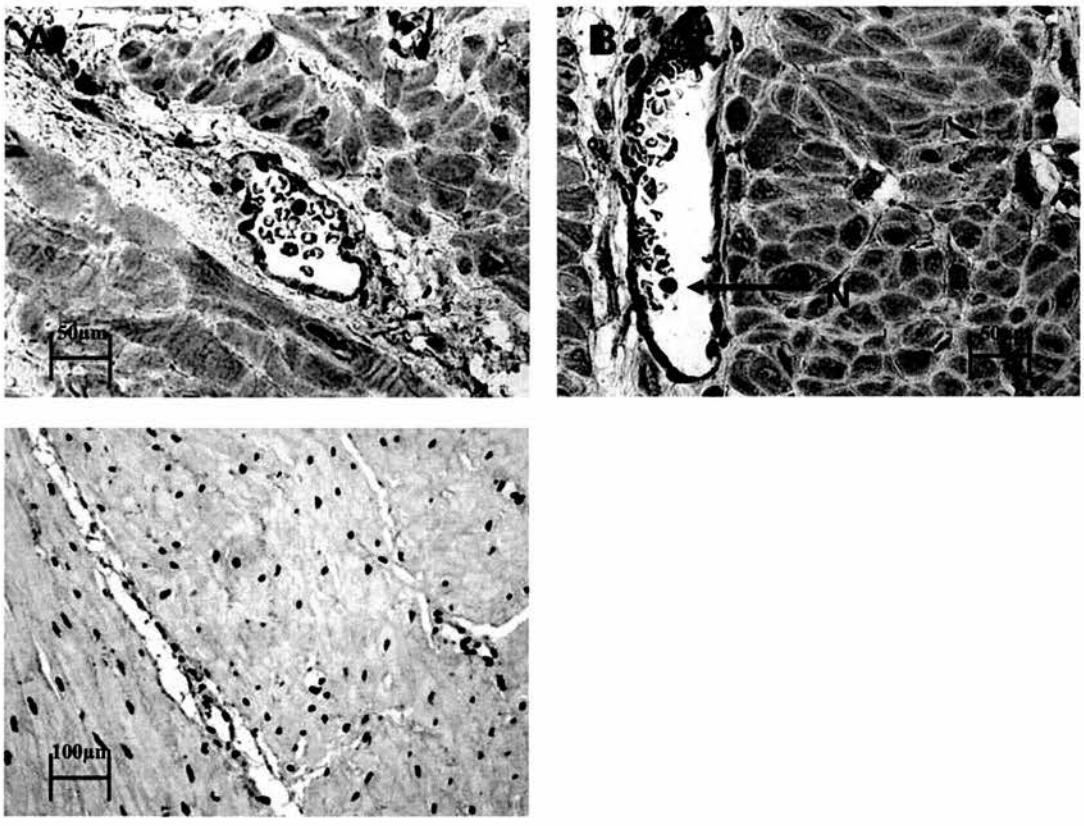
Figure 3.14 FPR2/ALX mRNA expression in labouring tissues
Relative mRNA expression of FPR2/ALX is not significantly different in the four labouring (L) tissues of myometrium, placenta, amnion and chorio-decidua. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11; p=0.26; Friedman test with Dunns post test.

3.3.4. FPR2/ALX localisation

FPR2/ALX immunostaining in term non-labouring and labouring myometrium revealed localisation of the receptor in uterine myocytes and in neutrophils (Figure 3.15). Immunofluorescence co-localised cells staining for neutrophil elastase and FPR2/ALX. In non-labouring myometrium, whilst staining was evident in myocytes, as in the immunohistochemistry, few cells staining for neutrophil elastase were observed. In labouring tissue, there were a greater number of cells that stained positively for neutrophil elastase and there was co-localisation of FPR2/ALX, confirming expression on neutrophils (Figure 3.16).

In placenta, FPR2/ALX immunostaining demonstrated localisation to the syncytiotrophoblasts in both non-labouring and labouring tissue (Figure 3.17).

In membranes, FPR2/ALX immunostaining demonstrated localisation to the single epithelial cell layer of the amnion, the chorionic trophoblast and the decidual cells in both labouring and non-labouring tissue. (Figure 3.18).



3.15 Immunolocalisation of FPR2/ALX in myometrium

Example of the immunolocalisation of the FPR2/ALX receptor in myometrium from a woman not in labour (A) and in labour (B). Staining is seen in myocytes and in endothelial cells surrounding the blood vessel. Stronger staining is seen in neutrophils (N). Negative control is shown below.

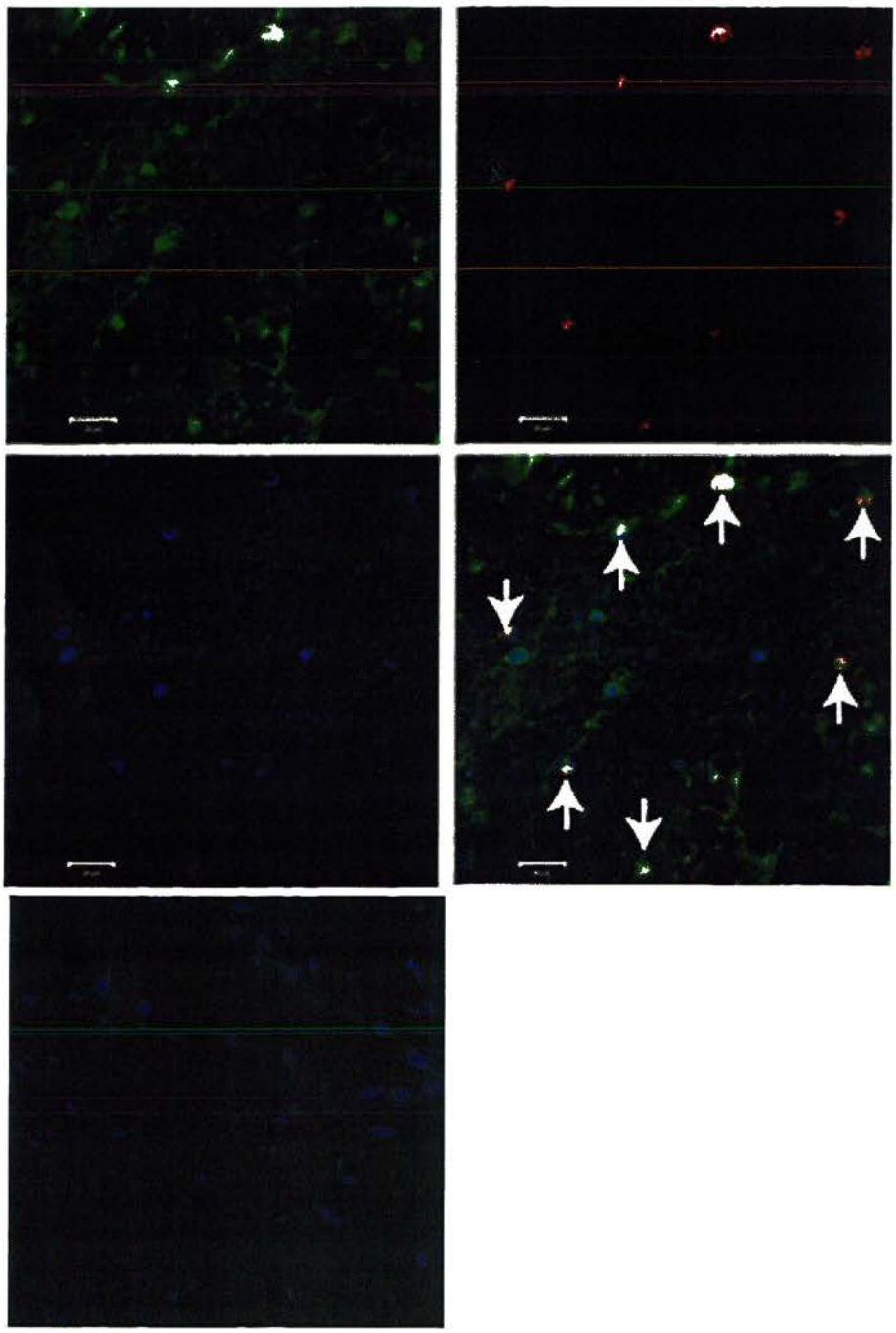
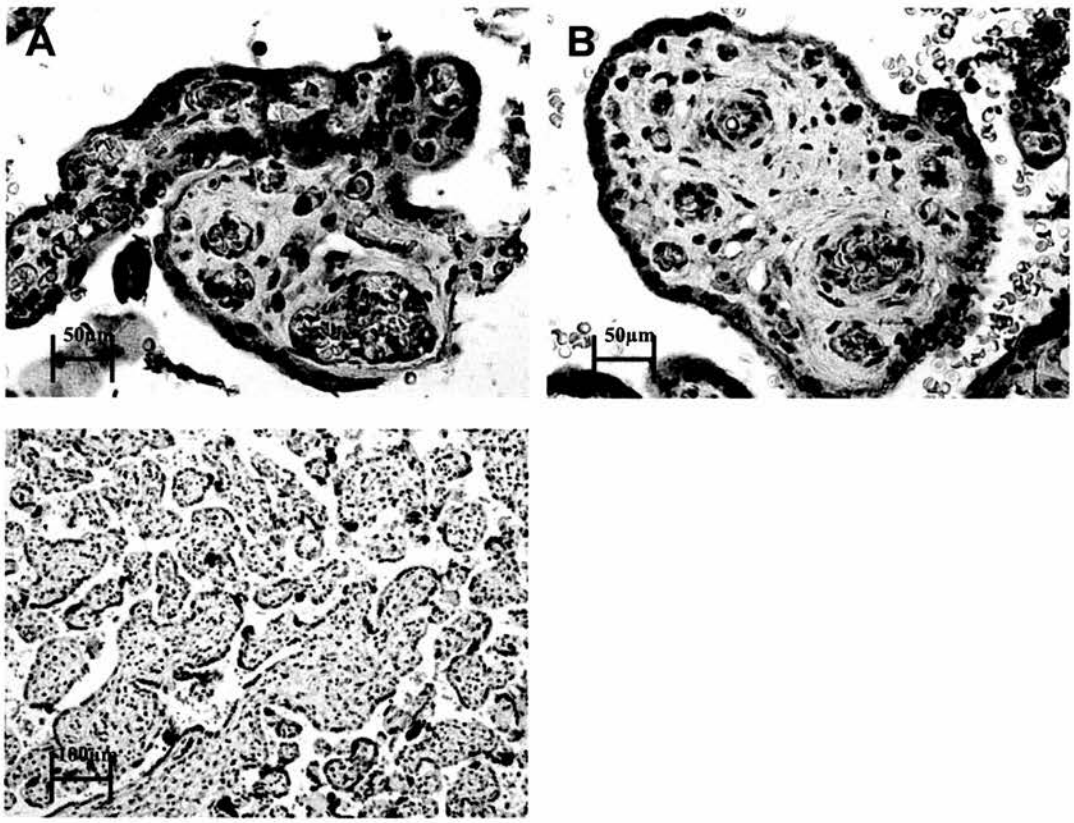


Figure 3.16 Co-localisation of FPR2/ALX and neutrophil elastase in myometrium

Example of the co-localisation of FPR2/ALX in labouring myometrium. Immunofluorescence staining shows that FPR2/ALX (green) is expressed in neutrophils (indicated by arrows) that have been stained with neutrophil elastase (red). Nuclear staining is shown in blue. Negative control (image below) shows only nuclear staining.



3.17 Immunolocalisation of FPR2/ALX in placenta

Example of the immunolocalisation of the FPR2/ALX receptor in placenta from a woman not in labour (A) and in labour (B). FPR2/ALX is localised to the syncytiotrophoblasts, with the strongest staining in the brush border contacting the maternal circulation. Staining is also observed in endothelial cells surrounding the blood vessels. Negative control is shown below.

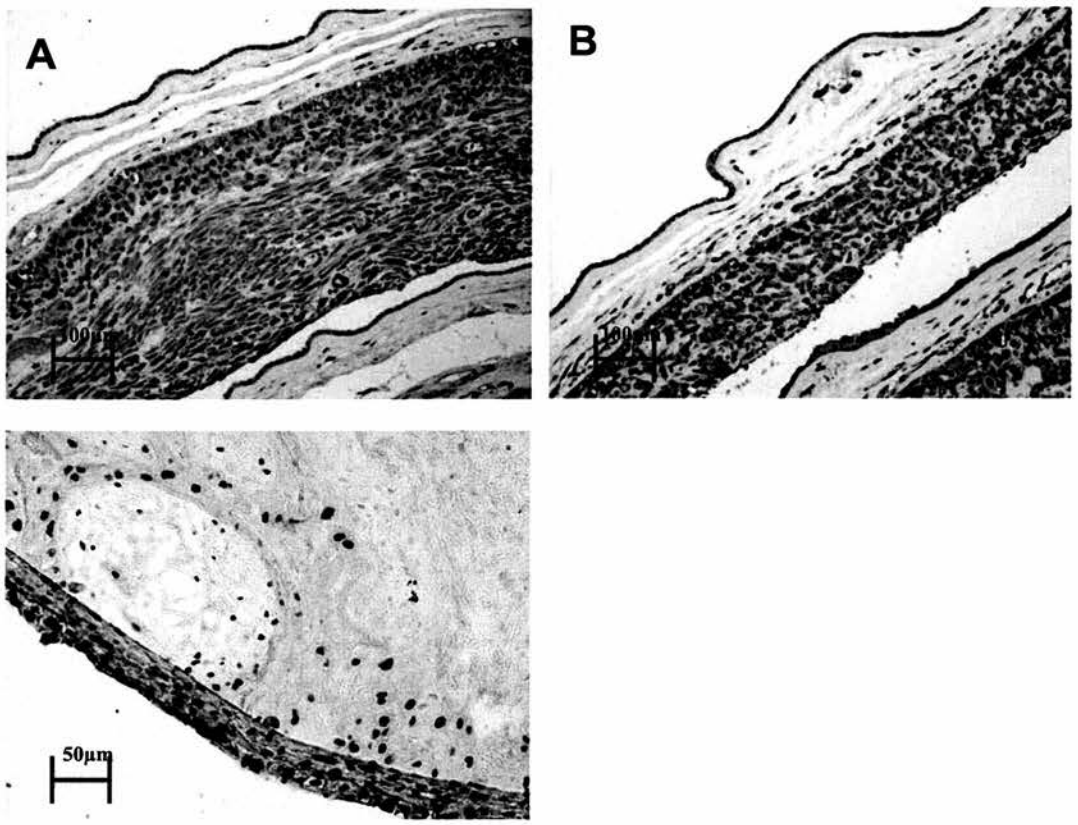


Figure 3.18 Immunolocalisation of FPR2/ALX in fetal membranes
Example of the immunolocalisation of the FPR2/ALX receptor in fetal membranes from a woman not in labour (A) and in labour (B). Negative control is shown below. Staining is seen in in the single epithelial layer of the amnion. There is also staining in the chorionic trophoblast and decidual cells.

3.4. DISCUSSION

The work in this chapter has shown that:

- LXA4 was detectable in circulating peripheral blood of both pregnant and non-pregnant women.
- Levels of circulating LXA4 were higher in pregnant women than in non-pregnant women, and increased during the third trimester from 24 weeks gestation to 37 weeks gestation.
- Levels of LXA4 were lower in women over 39 weeks gestation, whether in labour or not in labour, than in the cohort of women at 37 weeks gestation.
- Circulating LXA4 levels were no different in term labouring women than in gestation-matched non-labouring women.
- mRNA expression of ALOX5 was detectable in human term pregnant myometrium, placenta, amnion and chorio-decidua and was greater in labouring myometrium and chorio-decidua than in non-labouring.
- mRNA expression of ALOX15 was not detectable in the four studied tissues using the experimental techniques described, and only low levels of ALOX12 mRNA expression were detected.
- mRNA and protein expression of FPR2/ALX was detectable in human term pregnant myometrium, placenta, amnion and chorio-decidua, and relative mRNA expression was greater in labouring samples compared to non-labouring.
- Immunohistochemistry localised the FPR2/ALX protein to myocytes and neutrophils in myometrium, to syncytiotrophoblast in placenta and in membranes, to the epithelial cell layer of the amnion, the chorionic trophoblast and to decidual cells.

The work described in this chapter observes for the first time that levels of LXA4 are greater in pregnant than in non-pregnant women and increase towards term and has recently been published (Maldonado-Perez et al. 2010). The main described roles of LXA4 include effects on neutrophil trafficking (Serhan et al. 1995; Papayianni et al. 1996; Filep et al. 1999) and on reduction of secretion of pro-inflammatory cytokines

(Sodin-Semrl et al. 2000; Gewirtz et al. 2002). The onset of parturition may be characterised by an influx of inflammatory cells into the reproductive tissues (Osman et al. 2003) and an increase in secretion of pro-inflammatory cytokines (Winkler et al. 1998) and thus, given that LXA4 has, in other systems been observed to influence these processes, it is possible that LXA4 may have a role in modulating the onset or progress of normal and/or preterm labour.

Although the causes of preterm labour are not fully understood, infection is recognised as a major cause (Goldenberg et al. 2008; Norman et al. 2009), and inflammation without evident infection is also seen, and is associated with poor neonatal outcome (Shim et al. 2004). In the resolution of inflammation, it is important that an inflammatory response is brought to an appropriate end once it has served its purpose to protect the host from injury or microbial invasion. However, it is also important that an excessively large inflammatory response is not mounted in response to a minor or trivial insult, and in other systems in the human body, LXA4 has been implicated in having such a 'care-taking' role, in limiting and controlling an inflammatory reaction where a large response is not necessary. Thus, it may be that LXA4 is involved in controlling inappropriate inflammatory responses that may result in the escalation of inflammation and preterm labour, as the more advantageous result would be continuation of the quiescence of pregnancy. This concept would fit with the observed increase in circulating levels of LXA4 during pregnancy to term.

In my study, levels of LXA4 in the circulating blood were higher in the cohort of women at 37 weeks gestation than in the two cohorts of women who had blood taken around the time of delivery, either in labour or not in labour. Although 37 weeks gestation is considered term, most women do not deliver until closer to 40 weeks gestation. It would appear that there may be a fall in circulating LXA4 levels as labour approaches. However, there was no difference in women of matched gestation who were labouring spontaneously compared to those who were yet to labour. It would be interesting to confirm these results by taking longitudinal samples from a group of women at regular intervals during the third trimester until the point of

delivery, as it would give a more reliable answer than comparing non-matched cohorts, as my study has done. If this were to confirm that levels of LXA4 do reach a peak a few weeks before labour and then begin to fall, it would then be interesting to examine if this were the case with women who laboured preterm. This would be a very useful finding clinically, as it could potentially be used as a biomarker for estimating the time of onset of labour.

Whilst preterm labour is to be avoided, timely delivery of a term infant is, of course, necessary. Again, the concept of LXA4 acting as a 'brake' on inappropriate inflammation fits with the results described in this chapter in that there is no increase in circulating levels towards the end of pregnancy, and in fact, a fall at the very latter stages of pregnancy. It is not known what pathways stimulate this increased secretion of LXA4 in pregnancy, but it would appear that at term these pathways result in no further increase in production of LXA4, perhaps allowing a potential 'brake' to be eased off.

Circulating LXA4 levels in labouring women at term were no different from levels in gestation-matched women who were yet to labour. Our group have also shown that LXA4 is released from term myometrial explants with no differences between labouring and non-labouring samples (Maldonado-Perez et al. 2010). This suggests that either labour induces no additional release of LXA4 by myometrium, or that any additional release was matched by breakdown in this system. The explant system differs from in vivo conditions in that there is a lack of other tissues or cells and thus it is possible that the experimental conditions do not reflect actual in vivo conditions. For example, one of the steps in the synthetic pathway of LXA4 takes place in a neutrophil-platelet interaction, and this may be reduced or absent in the explant experiment as the explants are no longer in contact with a blood supply from which neutrophils and platelets are derived. Therefore it may be difficult to draw conclusions on whether these results actually reflect myometrial production of LXA4 in labour. However, the results fit with the observed results in circulating blood in labour and non-labour, and provide further evidence that production of LXA4 is not increased during labour.

I have shown in this work that mRNA expression of ALOX5 was upregulated in labour in myometrium and chorio-decidua, suggesting increased activity in the pathways of reactions which it catalyses. Brown et al have also demonstrated an increase in ALOX5 expression in labouring chorio-decidua, and also that expression was not increased in placenta or amnion (Brown et al. 1999). ALOX5 and ALOX12 mRNA expression have been observed in baboon intrauterine tissue, including myometrium, cervix and chorio-decidua (Smith et al. 2001), and there was no difference in labouring expression compared to non-labouring in myometrium. ALOX5 mRNA expression was increased in labouring baboon decidua, but decreased in the labouring chorion and decreased in cervix towards term (Smith et al. 2001) and ALOX12 expression was unchanged in labouring and non-labouring tissues. Thus there are some discrepancies in the published data, but overall, changes do appear to occur both towards the end of gestation and during parturition and therefore there are implications for the role of the proteins which are products of the pathways these enzymes catalyse. The lipoxygenase enzymes are also involved in the pathways that synthesise leukotrienes and prostaglandins, proteins with established roles in parturition, but LXA4 may also be a candidate for altered production or function.

FPR2/ALX expression was increased in labour in all four of the studied tissues, thus providing a mechanism by which the effects of LXA4 may be amplified, whether the actual levels of lipoxin are increased or not. It is notable in some of the groups, particularly in amnion, that there were wide ranges of relative mRNA expression of FPR2/ALX in the group. This may be for a number of reasons. Firstly, natural variation between individuals is to be expected. Secondly, the non-labouring women were delivered prior to the time that they would have gone into labour spontaneously, and at a presumably variable time from that point. It may be that the closer to natural labour a woman is may affect tissue expression of FPR2/ALX mRNA. Thirdly, the sites from which the membrane biopsies were taken were randomly selected. It is recognised that a zone of weakness overlying the cervix exists in fetal membranes at term (McLaren et al. 1999), characterised by thinner membranes, increased MMP-9 and decreased TIMP3 (El Khwad et al. 2005). It may

be that the greater variation in expression seen in the amnion biopsies is due to natural variation dependent on distance from this zone of weakness. The studies which have demonstrated this zone of weakness in the membranes identified the area overlying the cervix prior to delivery and then took biopsies at carefully determined distances from this zone (El Khwad et al. 2005; El Khwad et al. 2006). It would be interesting to perform a similar experiment looking at expression of FPR2/ALX to determine any regional differences in its distribution within the membranes, as this may also provide further clues as to its potential function within pregnancy and labour.

The elevated levels of circulating LXA4 in pregnancy, which increase to term, followed by an upregulation of its receptor during parturition itself, suggest a role for LXA4 within this process. It may be that dysfunction of the pro-resolution systems results in abnormalities of labour, for example, preterm labour, or prolonged labour at term. Currently there is no universally effective treatment for arresting preterm labour, and indeed is has been observed that attempting to do so and leaving the fetus in a pro-inflammatory mileu may in fact do more harm than good . The concept of using anti-inflammatory agents in the treatment of preterm labour therefore excites interest, and rodent models using anti-inflammatory agents such as IL-10 (Terrone et al. 2001) and PGJ2 (Pirianov et al. 2009) demonstrating dual effects of inhibiting labour and providing fetal neural protection are encouraging. It is possible that LXA4 may prove to have therapeutic value in the treatment of preterm labour.

These data suggest a role for LXA4 in human labour, and in order to consider how it may potentially be exploited to be used for therapeutic purposes in abnormal labour, more information on its effects in human tissues and the pathways by which it functions is needed. Work from other members of our group has explored the effects of LXA4 on myometrial explants treated with LPS to induce inflammation (Maldonado-Perez et al. 2010). LPS treatment increased the relative mRNA expression of the pro-inflammatory cytokines IL-6 and IL-8. However, in explants co-treated with LXA4, whilst there was still increased mRNA expression of IL-6 and IL-8 than in vehicle-treated explants, this was markedly reduced by the presence of

LXA4. Thus, a potential functional effect of LXA4 in parturition has been demonstrated in an in vitro environment. The next step, described in the following chapter will be to further explore how LXA4 may function in the myometrium.

3.4.1. Summary

LXA4 may have a role within human parturition. Levels of the mediator itself did not appear to be increased in term parturition, but expression of its receptor was increased, thus providing a mechanism for greater activity. Early work by our group would appear to show a mechanism by which LXA4 may modulate other mediators which have been demonstrated to be involved in the process of labour. In the next chapter, it is described how this experiment is developed to explore other pathways by which LXA4 may exert its effects.

4. Microarray of myometrium treated with LPS and lipoxin A4

4.1. INTRODUCTION

The work in the previous chapter described the potential role of LXA4 and its receptor FPR2/ALX in the process of human parturition. As was discussed, members of our group have published data demonstrating modulation by LXA4 of the pro-inflammatory response to LPS in myometrial explants (Maldonado-Perez et al. 2010). LPS increased mRNA expression of key pro-inflammatory cytokines in myometrial explant culture, whilst co-culture with LXA4 significantly reduced the effect of LPS on increased expression. The cytokines studied were IL-6 and IL-8. IL-1β was also studied and whilst a trend towards the same pattern was observed, this inhibition in IL-1β was not significant.

Microarrays examine the expression of a very large number of genes in a single experiment, providing a wide-ranging survey of the alterations in gene expression when comparing two or more samples. The data produced may uncover hitherto unrecognised involvement of certain genes within a biological process, opening up avenues of investigation that were previously unexplored. Functional analysis of the differentially expressed genes then allows the investigator to identify pathways by which a biological process may be progressing, to make links between interacting genes and to potentially develop ways of manipulating that biological process.

Several investigators have used microarray technology and functional genomics to explore the molecular mechanism of human parturition in labouring and non-labouring reproductive tissues (Aguan et al. 2000; Esplin et al. 2005; Havelock et al. 2005; Bollopragada et al. 2009; Mittal et al. 2010). Whilst there is some agreement across studies on some differentially expressed genes, there is also a lack of uniform gene expression signature, probably due to differences in experimental design, sample number and tissues types and the platforms used. Two recent functional genomic studies have reported that human parturition is characterised by inflammatory pathways, particularly those involving cellular trafficking (Bollopragada et al. 2009; Mittal et al. 2010).

Human parturition is a complex process, with many interacting pathways. LXA4 has been shown to have an effect on the expression of two key cytokines involved in parturition, as described above. The aim of the work described in this chapter is to further explore what effects LXA4 may have on human myometrium, and in particular within the process of inflammation and parturition.

An explant experiment using myometrial tissue from term, pregnant non-labouring women was performed. LPS treatment was used to induce inflammation in the explants, whilst other explants were treated with LXA4 or LPS and LXA4 together. Subsequently, a microarray was performed on RNA extracted from the explants to observe the effects of the treatments on gene expression within the samples. The results of the microarray were compared to published comparisons of the myometrial transcriptome in labouring and non-labouring tissue. Results of the microarray were validated using quantitative RT-PCR for a selected number of specific genes and additionally, relative gene expression for the selected genes was determined in labouring and non-labouring myometrial tissue samples.

4.2. METHODS

4.2.1. Patient recruitment

4.2.1.1. Samples for use in explant tissue culture:

Five non-labouring women were recruited to donate myometrial biopsies at elective caesarean section and informed, written consent was obtained as described in section 2.2.1. The women were all undergoing elective caesarean section (c/s) in an uncomplicated, singleton pregnancy, at term. Characteristics of the recruited patients are found in table 4.1.

Sample name	Age	Parity	Gestation (weeks+days)	Indication for delivery
B040	40	1+2	38+5	Previous c/s
B041	37	0+0	38+0	Placenta praevia
B044	24	0+0	39+5	Breech presentation
B045	32	1+1	39+0	Previous c/s
B047	22	1+0	39+4	Previous c/s

Table 4.1
Characteristics of the patients whose myometrial biopsies were used for the microarray experiment.

4.2.1.2. Samples for use in comparison of labouring vs non-labouring myometrium by quantitative RT-PCR

Groups of labouring (n=11) and non-labouring women (n=11) were recruited and informed, written consent was obtained to provide tissue samples as described in section 2.2.1. Inclusion and exclusion criteria for these groups are described in section 2.2.2.1.

4.2.2. Tissue collection

4.2.2.1. Samples for use in explant tissue culture

Myometrial biopsies were taken from the upper margin of the transverse lower uterine segment incision, after delivery of the baby, as described in section 2.1.5.1. Biopsies were taken by the operating surgeon using either curved mayo scissors or a scalpel and were full thickness, up to 1cm width and 2cm length. The biopsies were placed into RPMI culture medium, transported to the laboratory and used immediately in the explant culture experiment.

4.2.2.2. Samples for use in comparison of labouring vs non-labouring myometrium by quantitative RT-PCR:

Samples of myometrium were taken at the time of elective or emergency caesarean section, as described in section 2.1.5.1.

4.2.3. Tissue culture

The myometrial biopsies were washed three times with PBS and then the decidua was dissected off and the tissue was divided into explants of approximately 1-2mm3 using scissors. The explants were placed into fresh RPMI medium in an incubator at 37°C and gassed with room air overnight in order to starve the explants of any remaining blood that may have been residual after washing. The following morning, explants were placed into RPMI culture medium supplemented with penicillin/streptomycin (P/S) (5000 u/ml, 5000μunits/ml) and containing one of the following treatments:

1. **Vehicle** (sample numbers 1-3)
   The LXA4 used was suspended in 100% ethanol, therefore 100% ethanol was added to the culture medium to give the same concentration as in the samples treated with LXA4.
2. **Lipopolysaccharide (LPS) 100ng/ml** (sample numbers 4-6)
3. **LXA4 100nM** (sample numbers 7-9)
4. **LXA4 100nM and LPS 100ng/ml** (sample numbers 10-12)

In each experiment, the explants and culture medium were placed into wells on a 24 well plate. In each well, 1 ml of culture medium was used and 2 explants were used. For each treatment 3 triplicate wells were used. In wells in which LPS was used, the LPS was added after 30 minutes of pre-treatment with either LXA4 or vehicle and then the plates returned to the incubator.

The plate was placed into culture in an incubator at 37°C for 8 hours. After this time, the plate was removed from culture. Myometrial explants were removed from culture and placed into a pre-weighed 2ml Eppendorf tube and immediately placed onto dry ice to snap freeze the sample. Samples were then transferred for storage at -80°C until further analysis. Forceps were washed between each separate well.

4.2.4. RNA isolation

RNA was extracted from the explants using the method described in Section 2.4.1. RNA quality and concentrations were checked using a Nanodrop 1000 spectrophotometer (ThermoScientific, Wilmington, DE, USA). Absorbancy at 260nm and 280nm was measured and the concentrations given in ng/µl. The RNA extraction and quality check was performed by Sharon Battersby, whose assistance is gratefully acknowledged.

4.2.5. Microarray

Following RNA extraction the samples were sent to Turku Centre for Biotechnology, where a microarray was performed using an Illumina Human HT-12 v.3 Expression Beadchip. This chip contains probes for more than 48000 measurement features and the expression value for each of the probe types is calculated from approximately 15 measurements. The following section describes their methods.

4.2.5.1. *RNA quality assessment, hybridisation and normalisation*

Following quality assessment of the samples by Experion capillary electrophoresis assay, the best quality of the three replicates in each group was selected and hybridised, leaving a total of 20 samples, i.e. 5 patients and 4 treatment groups.

4.2.5.2. *Intensity signal values*

For each array feature, a signal intensity value was detected. In any typical hybridised sample, most array features yield very small intensities and just a few features have high intensities. This is illustrated in Figure 4.1.

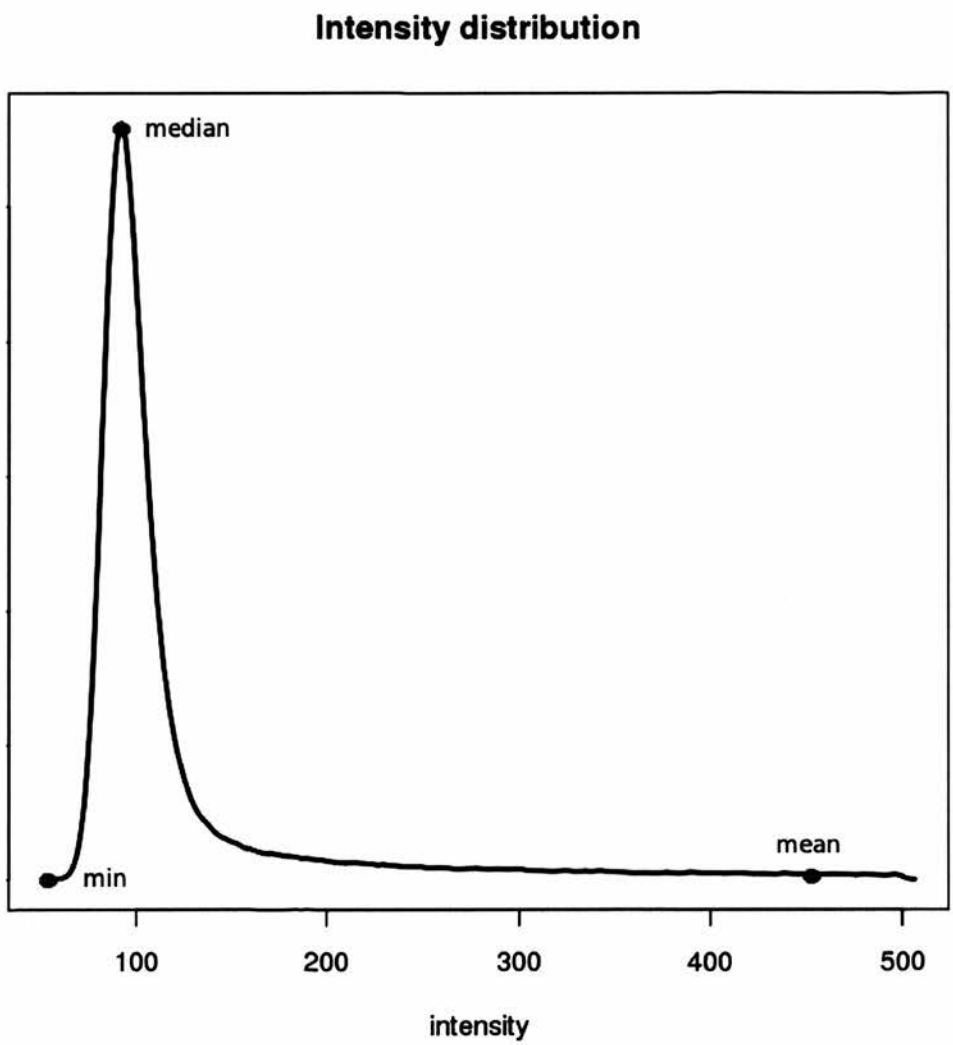


Figure 4.1 Intensity distribution curve

Curve describing the intensity distribution of a typical hybridised sample. Most array features yield small intensities (close to background), evidenced by the median peak towards the left of the curve and only a few features have high intensities.

4.2.5.3. Normalisation

If there are large variations in the distributions between samples, this can indicate problems with quality. Normalisation compensates for minor variations in distribution and brings the expression values into the same register so that the samples can be compared to each other. The data were normalised to remove variation between samples caused by non-biological reasons ie minor variations caused by experimental processes. The data were normalised applying the quantile normalisation. First, each sample's measurement features are arranged in ascending order based on expression value. Then the expression values are replaced with their mean on every sample and the means are arranged in the original order based on the expression values.

4.2.5.4. Sample relations

Sample relationships were studied by means of correlation and cluster analysis. Each sample was compared with the other samples, taking all measurement features into consideration. In the analysis, sample similarity is described on a scale of 0 to 1, where 0 means absolutely no correlation, and 1 means perfect correlation.

Samples were clustered with Pearson's metrics to group the samples according to their general similarity when all array measurements were taken into account.

4.2.5.5. Group comparisons and identification of differentially expressed genes

The following groups were compared to detect differentially expressed genes between the two groups, which are given the following titles:

- LPS vs Vehicle
- Lipoxin vs Vehicle
- LPS&Lipoxin vs Vehicle

- LPS&Lipoxin vs LPS
- LPS&Lipoxin vs Lipoxin

The statistical package used was R package Limma. This package uses linear modelling and empirical Bayesian methods to assess differential expression in gene expression data. Initially a linear model is created for each measurement feature of the array to give an estimate of the group average expression. This model is then used to produce the modified t-test p-values of the comparisons between different groups. Thus, the smallest p-values result from comparisons where the samples within each group (ie biological replicates) are similar but the values between sample groups differ.

Statistical t-tests were performed separately for each measurement feature and because a very large number of tests are performed, calculations are built in for the potential false positive findings that may statistically result. Limma therefore calculates an adjusted p-value which is an FDR (false discovery rate) corrected p-value.

The fold changes and corrected p-values from the statistical comparisons were used as filtering criteria to create lists of genes which had the strongest evidence for being differentially expressed between the two groups. Thresholds were set that resulted in a reasonable number of differentially expressed array features.

4.2.5.6. Volcano plots

Volcano plots were used to visualise the relationships between fold change and p-values of the filtered array features in the compared groups. The log fold change between the two groups being compared is plotted on the x-axis and the log10 of the p-values is plotted on the y-axis for each array feature. Dotted lines represent the filtering threshold. Upregulated array features are represented by red dots, downregulated array features are represented by green dots and array features in which no significant change was found when comparing the two groups are represented by black dots. The greater the fold difference between the two groups, the further away the dots fall from x=0 and the more significant the p-value is, the

further away the dot falls from y=0. Thus for each comparison the scattering of the plot demonstrates what effect the given treatment has had, with tighter scattering and fewer coloured dots indicating a lesser effect, and broader scattering and more coloured dots indicating more effect. Additionally, a preponderance of red dots is suggestive of a stimulatory effect of the treatment, whereas a preponderance of green dots would suggest an inhibitory effect on gene expression in general.

4.2.5.7. Clustering by Principal Component Analysis (PCA)

PCA was also used to visualise the sample relationships in three dimensional space. PCA analysis orders objects so that similar objects are placed close to one another and dissimilar objects are placed further apart.

4.2.5.8. Heatmaps

Heat maps use Pearson's metrics to cluster the samples and filtered features. The clustering is based on the general expression measurement similarity. In the plots, each row represents a differentially expressed feature, and each column represents one sample. Red colour indicates high expression and green represents low expression.

4.2.5.9. Functional analysis

Functional analysis of the data was performed using MetaCore from GeneGo. This tool is based on a manually curated database of published protein-protein, protein-DNA and protein compound interactions, metabolic and signalling pathways, supported by proprietary ontologies and controlled vocabularies. The data gathered from the array is applied to algorithms developed from these databases to identify networks and canonical pathway maps relevant to the experiment.

4.2.5.10. Validation with quantitative RT-PCR

Five differentially expressed genes were selected from the array experiment LPS vs vehicle and the results validated using quantitative RT-PCR. Discussion on the criteria used to select the five validated genes is found in the corresponding results section of this chapter.

cDNA was synthesised from the explant RNA samples, as described in section 2.4.

Quantitative RT-PCR was performed as described in section 2.5 . The primers and probes used to determine expression of the genes of interest were TaqMan Gene Expression Assays. These are commercially available predesigned, preformulated primer and probe sets specific for the selected gene.

The TaqMan Gene Expression Assays used were:

- E-selectin Hs00950401_m1
- Colony stimulating factor 3 (CSF3) Hs00738432_g1
- Intercellular adhesion molecule 1 (ICAM1) Hs00164932_m1
- Matrix metallopeptidase (MMP1) Hs00899658_m1
- Chemokine (C-C motif) ligand 2 (CCL2) Hs00234140_m1

The reaction mix used gave a final volume of 15µl per well, containing 7.5 µl 2x Express Mastermix, 0.75µl primer/probe gene expression assay mix, 0.225µl 18S primer/probe assay mix, 0.375µl cDNA and 6.15µl RNase-free, DNase-free sterile water.

4.2.5.11. Labouring vs non-labouring comparison

It was of interest to examine whether the genes upregulated by LPS were also upregulated in labour. Therefore, relative mRNA expression of the five abovementioned genes was also determined in samples of labouring (n=11) and non-labouring (n=11) myometrium. The samples used were the same as those used in the work in Chapter 3. RNA was extracted and cDNA manufactured as described in section 2.5.3. Quantitative RT-PCR was performed on these samples according to the method in section 2.5.4 and the assays and reaction mixes used are described in the previous paragraphs.

4.2.5.12. Data analysis

A Mann-Whitney test was performed to compare relative gene expression in samples of labouring and non-labouring myometrium. To compare the relative levels of mRNA expression between the treatment groups, a Repeated Measures ANOVA with Bonferroni post test was used. All statistical calculations were carried out using GraphPad Prism 5.02 (GraphPad software, CA, USA).

4.3. RESULTS

4.3.1. RNA quality control

Assessment of RNA quality prior to microarray analysis demonstrated that all samples were of good quality and concentrations were within the range required by the laboratory at the Turku Centre for Biotechnology to perform the microarray; data are shown in Table 4.2. Once at the Turku laboratory, the 60 samples were screened again and the best samples from each group were selected for the hybridisations. There were no reported problems in the amplification and labelling procedures and the overall quality of the hybridized samples was good.

	B040		B041		B044		B045		B047	
	Conc (ng/µl)	260/ 280	Conc (ng/µl)	260/ 280	Conc (ng/µl)	260/ 280	Conc (ng/µl)	260/ 280	Conc (ng/µl)	260/ 280
1	200.29	2.11	146.04	2.1	95.53	2.09	228.12	2.13	123.21	2.04
2	110.1	2.13	171.4	2.1	58.21	2.11	223.7	2.09	179.75	2.02
3	156.6	2.09	102.9	2.06	121.73	2.09	235.08	2.11	101.19	2.03
4	188.32	2.09	123.32	2.1	141.82	2.09	195.2	2.09	100.03	2.02
5	293.35	2.11	142.19	2.1	137.74	2.08	178.89	2.08	128.45	2.06
6	109.31	2.1	179.23	2.11	46.41	2.02	193.22	2.13	41.52	2.01
7	202.83	2.13	168.52	2.08	111.86	2.1	194	2.11	92.49	2.05
8	160.42	2.12	93.6	2.11	130.28	2.08	169.67	2.07	101.71	2.05
9	226.41	2.12	157.91	2.13	89.71	2.14	191.59	2.07	158	2.07
10	187.15	2.1	148.3	2.09	142.8	2.1	139.68	2.09	130.18	2.06
11	204.68	2.1	114.09	2.13	73.92	2.1	272.3	2.08	134.2	2.04
12	298.48	2.11	153.27	2.1	82.29	2.14	211.04	2.08	189.47	2.05

Table 4.2 RNA sample concentrations and qualities

Table shows RNA concentrations and 260/280 absorbance ratios demonstrating good quality and adequate concentrations of RNA were obtained from each sample. Sample numbers 1-12 refer to the well numbers, as described above.

4.3.2. Hybridisation intensity over the chips

Table 4.3 shows the minimum, mean and maximum intensities of the normalised samples.

Minimum	Median	Mean	Maximum
48	85	481	38889

Table 4.3 Sample intensity summary

Most of the features of the array are not expressed in most experiments, thus the median value can be regarded as a rough measure of the background.

These intensity distributions are displayed as box plots before and after normalisation in Figure 4.2. The chips were evenly hybridised and therefore, as can be seen in the figure, there was little need for large normalisation.

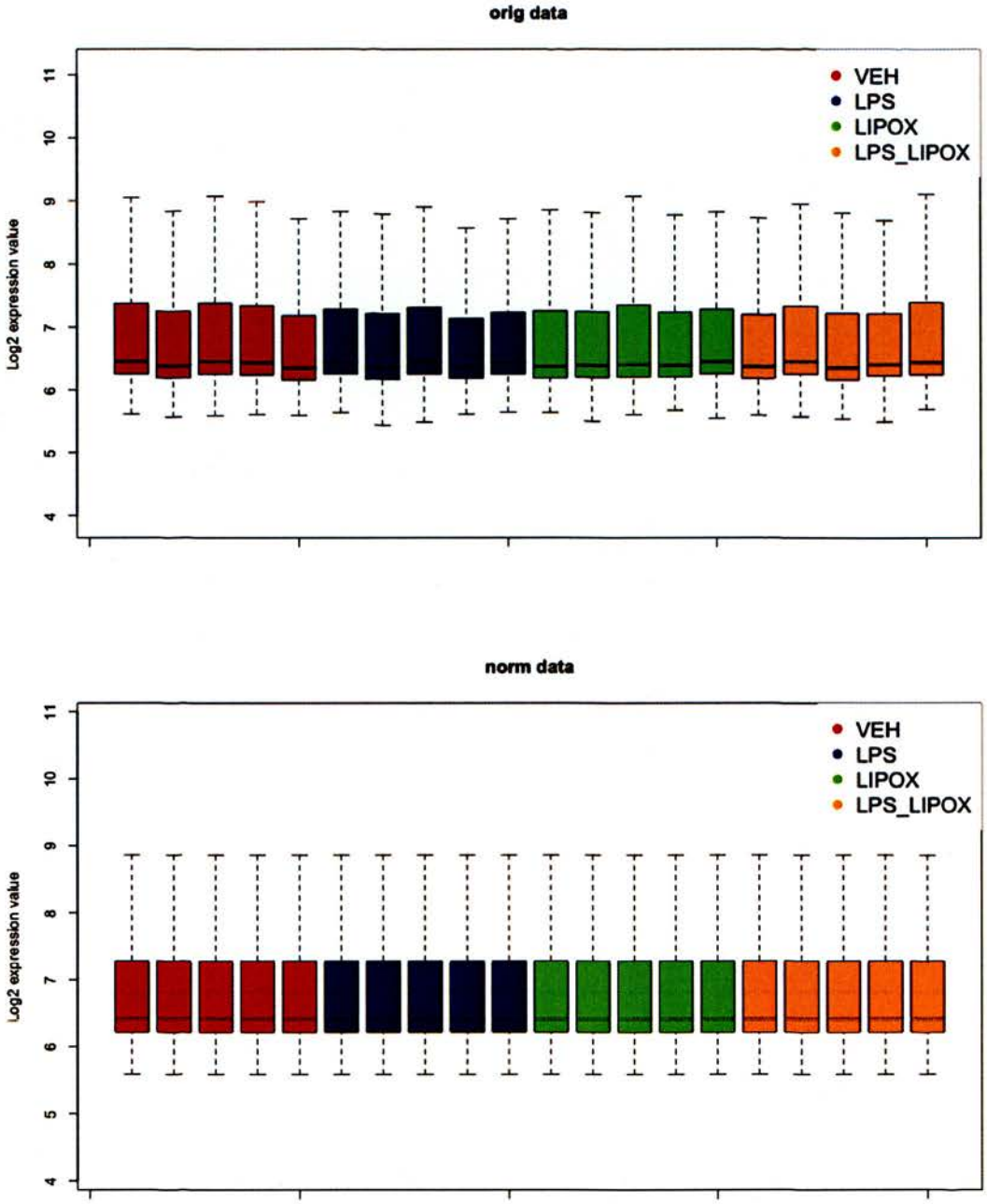


Figure 4.2 Expression intensity value distributions
Expression intensity value distributions (log2-transformed) as box plots for unnormalised and normalised data.

4.3.3. Sample relations

Groupwise minimum and mean correlations are given in Table 4.4. In samples that are biological replicates, correlation of at least 0.95 is expected, so the data in Table 4.4 demonstrate good correlation within the groups.

Group	Minimum Correlation	Mean Correlation
Vehicle	0.976	0.98
LPS	0.956	0.975
Lipoxin	0.967	0.979
LPS and Lipoxin	0.963	0.972

Table 4.4

Groupwise minimum and mean correlations

The correlation values are illustrated in Figure 4.3.

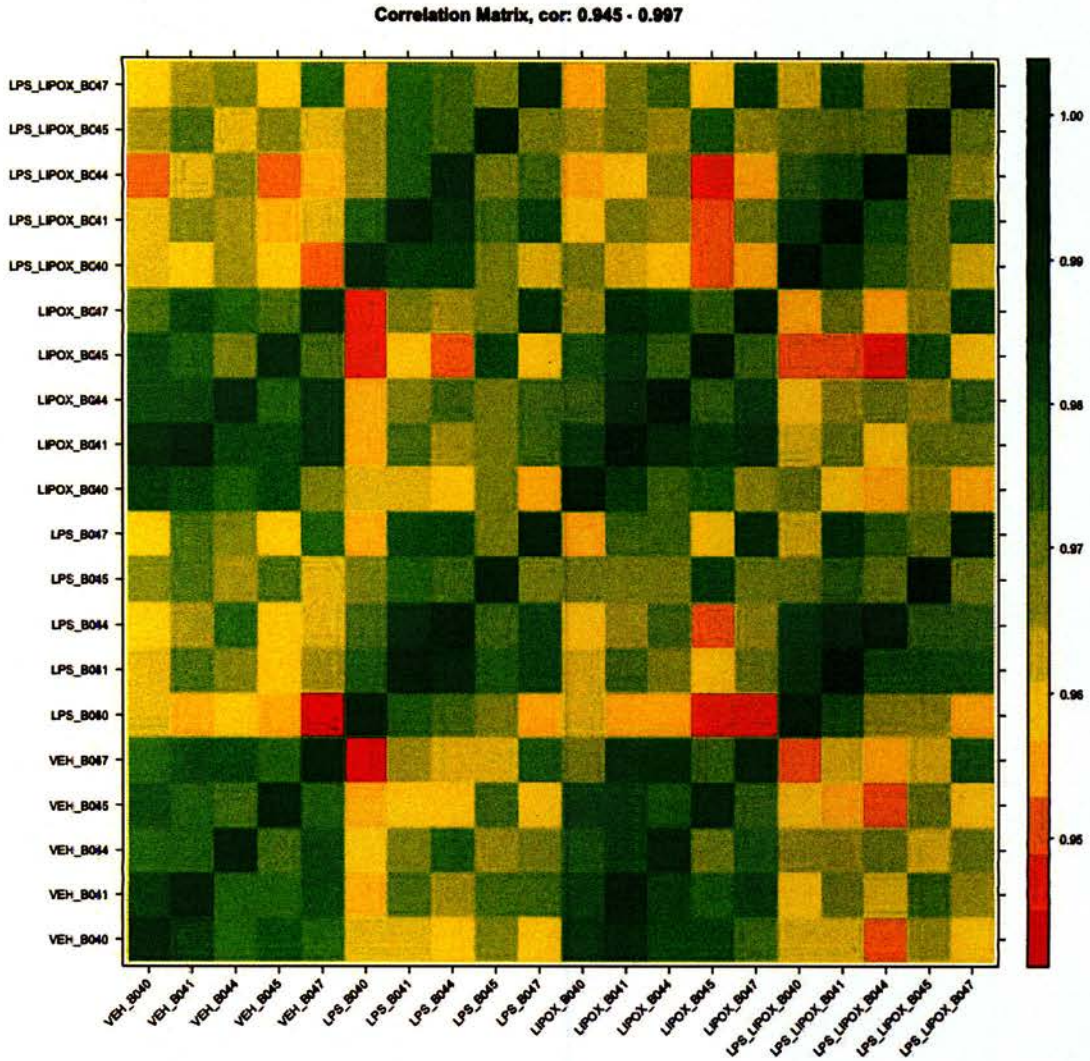


Figure 4.3

Sample correlations for normalised data.

Full figure legend follows on next page.

Figure 4.3
Sample correlations for normalised data.

Figure is illustrated on the previous page.

Correlation in this analysis is described on a scale of 0 to 1, where perfect correlation has the value 1 and absolutely no correlation has the value 0. In this diagram Value 1 is dark green, and Value 0 is dark orange, with a scale in between as illustrated by the bar to the right of the diagram. Each individual line represents a sample and the samples are placed in order by their experimental group, for example, the first five lines represent the 5 samples from the LPS&lipoxin group. Each individual column also represents a sample, placed in the same order as the rows, enabling comparison of each sample to every other sample. Where a sample is compared with itself, perfect correlation is observed; this can be seen by the line of dark green squares running diagonally from bottom left to top right. Correlations between samples in the same group i.e. biological replicates, are generally green, demonstrating that there was fairly good correlation between biological replicates. Where samples that were treated with LPS are compared with samples not treated with LPS, correlation tends to be toward the yellow/orange end of the spectrum, indicating that there were differences between the samples. However, where samples that were treated with lipoxin are compared with similar samples that were not treated with lipoxin, correlations tend to be high, suggesting that treatment with lipoxin has not demonstrated large differences in the samples.

Samples were clustered with Pearson's metrics to group the samples according to their general similarity when all array measurements were taken into account. This is visualised in a dendrogram in Figure 4.4.

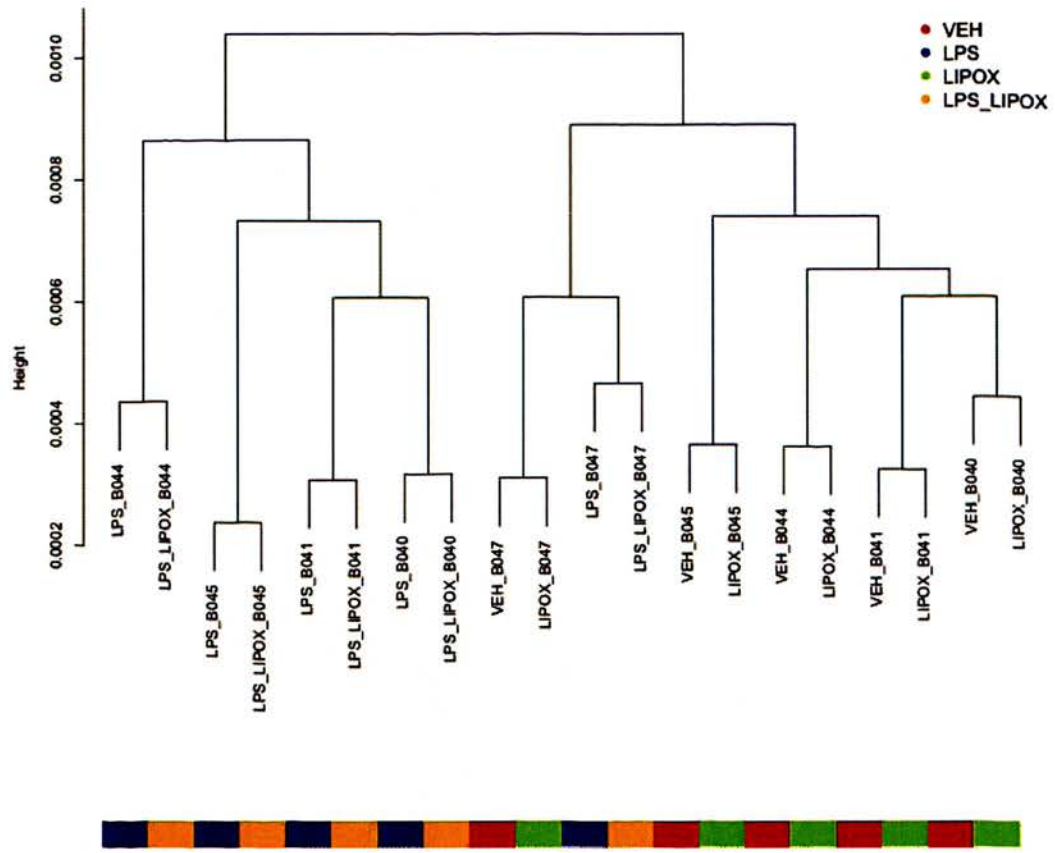


Figure 4.4

Hierarchical clustering for the normal data.

In this dendrogram the samples are placed according to their general similarity, when all array measurements are taken into account. Samples which are most similar are placed in the branches closest to each other. The dendrogram shows that the samples have generally clustered according to LPS treatment into two main clusters, and samples from the same individual cluster together within the two main clusters. This shows that LPS is the main differentiating feature between the samples and that the effect of LXA4 treatment was less significant than differences between biological replicates.

4.3.4. Group comparisons and identification of differentially expressed genes

Thresholds were set that resulted in a reasonable number of differentially expressed array features, and are displayed in Table 4.5. As could be expected from the

correlation and clustering analysis previously described, there were significant variations between the comparisons. Where the compared groups differed in LPS treatment, stricter threshold filters were used and these resulted in the filtered array features clustering according to treatment groups. However, where the compared groups did not differ in LPS treatment, the filtered array features clustered by sample individual as opposed to treatment group, suggesting that the differences in gene expression between individual patients is greater than the differences caused by the treatment.

	FC	logFC	PType	P	Tot	Up	Down
LPS vs Vehicle	2.0	1.0	FDR	0.001	294	228	66
Lipoxin vs Vehicle	1.2	0.26	P-value	0.05	102	40	62
LPS&Lipoxin vs Vehicle	2.0	1.0	FDR	0.001	293	231	62
LPS&Lipoxin vs LPS	1.2	0.26	P-value	0.05	96	64	32
LPS&Lipoxin vs Lipoxin	2.0	1.0	FDR	0.001	313	260	53

Table 4.5

Filtering summary table.

This table gives the values that were set as thresholds for producing the lists of differentially expressed genes. As can be seen, different thresholds were used for different comparisons, which reflects the effectiveness of the respective treatments in inducing differential gene expression.

4.3.5. Volcano plots

The following volcano plots (Figures 4.5-4.9) provide a graphical representation of the relationship between the fold change and the p-value for each individual array feature in each of the group comparisons. Where the difference between the two compared groups is treatment with LPS, a large number of dots fall outwith the

threshold lines, and there are more red dots than green, indicating a relatively large effect on gene expression brought about by LPS treatment and that the predominant effect is an upregulatory as opposed to a downregulatory effect on gene expression. Where the difference between the two compared groups is treatment with LXA4, fewer dots fall outwith the threshold lines, despite the thresholds having been set at lower levels in these groups. This illustrates graphically that LPS is having a large effect on gene expression in the myometrial explants, whereas LXA4 is having a much smaller effect.

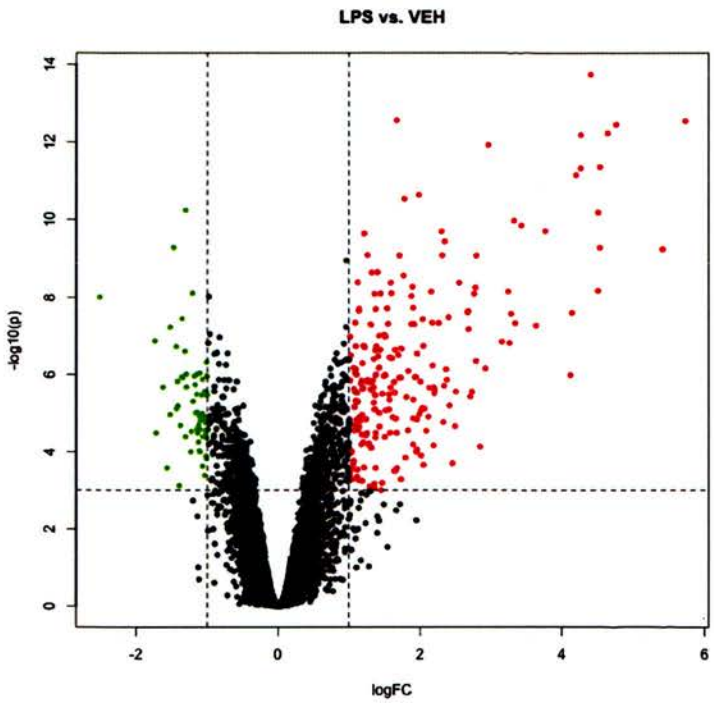


Figure 4.5

Volcano plot for LPS vs Vehicle

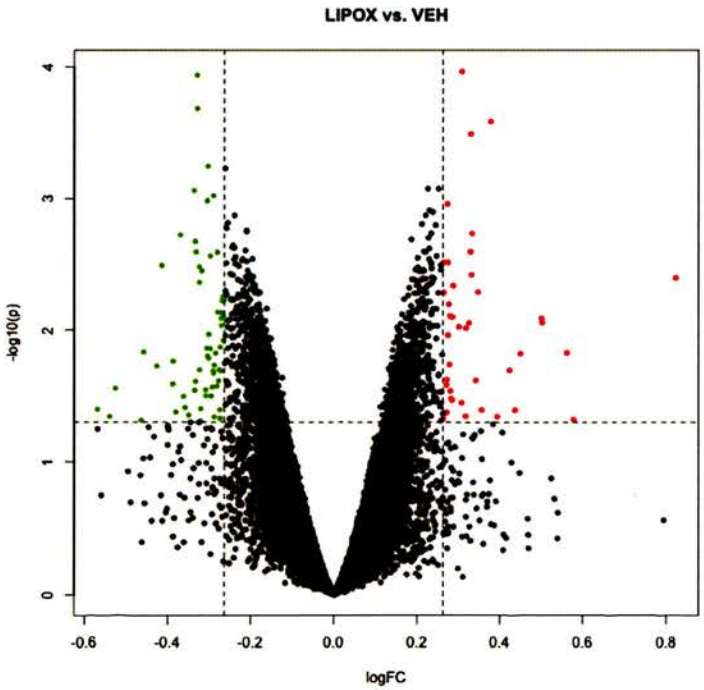


Figure 4.6

Volcano plot for Lipoxin vs Vehicle

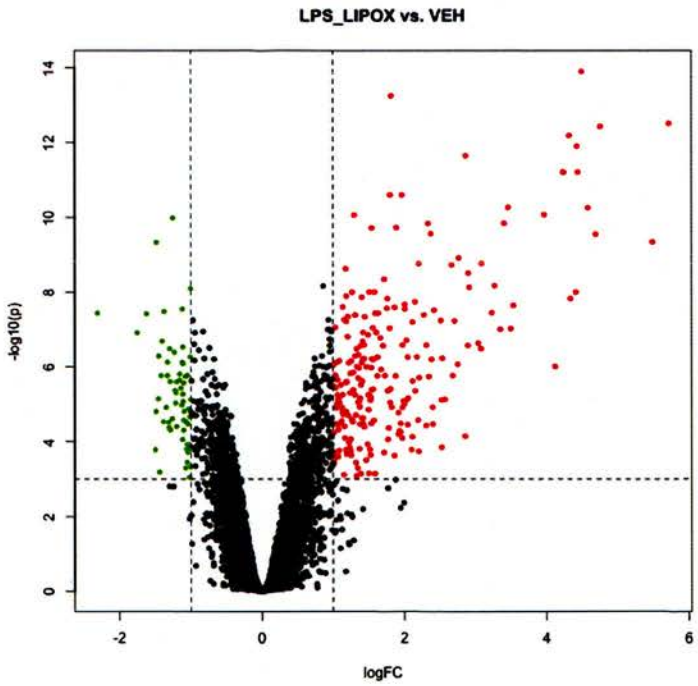


Figure 4.7

Volcano plot for LPS&Lipoxin vs Vehicle

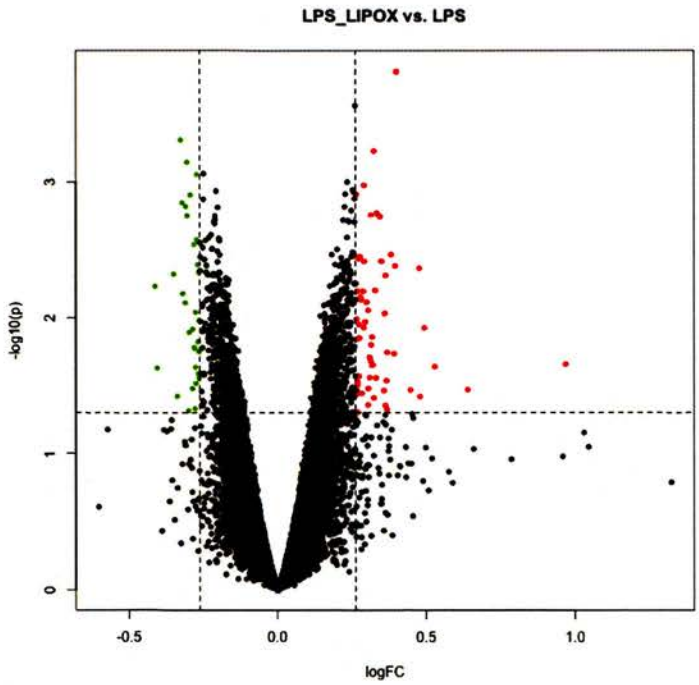


Figure 4.8

Volcano plot for LPS&Lipoxin vs LPS

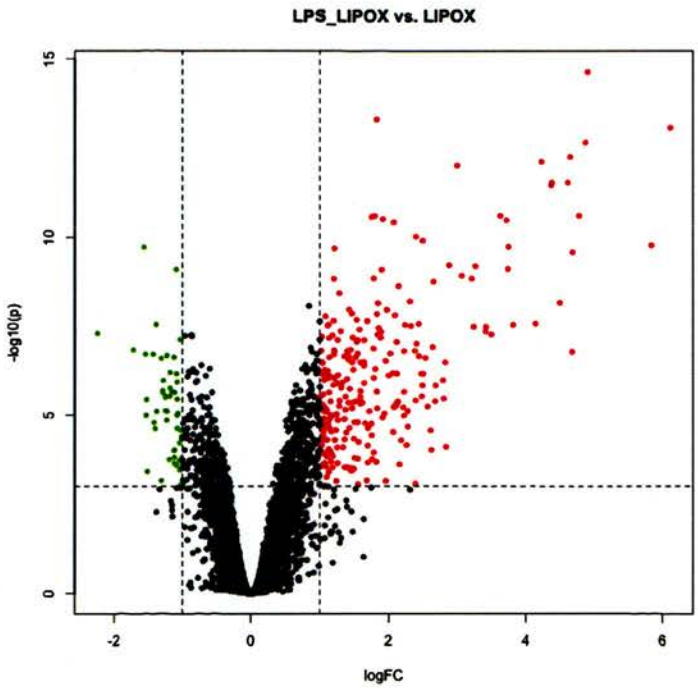


Figure 4.9

Volcano plot for LPS&Lipoxin vs Lipoxin

Figs 4.5 to 4.9 illustrate volcano plots for each of the comparison groups studied. The log10 of the p-values is on the y-axis and the logFC calculated for the comparison group vs base level group is on the x-axis. The filtering thresholds (as described in Table 4.5) are signified by dotted lines. Red dots signify upregulated array features and green dots signify downregulated array features. Figures 4.5, 4.7 and 4.9 are similar in that the two groups being compared differ in LPS treatment. Figures 4.6 and 4.8 are similar in that the two groups being compared differ in LXA4 treatment. Where the groups being compared differ in LXA4 treatment it can be observed that there are relatively few dots falling beyond the set thresholds. In contrast, where the groups being compared differ in LPS treatment, it can be observed that many more dots fall outwith the thresholds.

4.3.6. Principal Component Analysis (PCA) plots

The following PCA plots (Figures 4.10 to 4.14) use the data from the differentially expressed genes in each of the group comparisons to produce a three-dimensional visualisation of the similarity of the samples. Similar objects are placed close together and dissimilar objects are placed further apart. Where group comparisons differ because of LPS treatment, it can be seen that the PCA plot draws into two distinct clusters, separating the LPS-treated group and the non-LPS-treated group. However, in the group comparisons where the difference is LXA4 treatment, the dots do not draw into clusters, suggesting that variability between individual patients in the study is greater than any difference induced by LXA4.

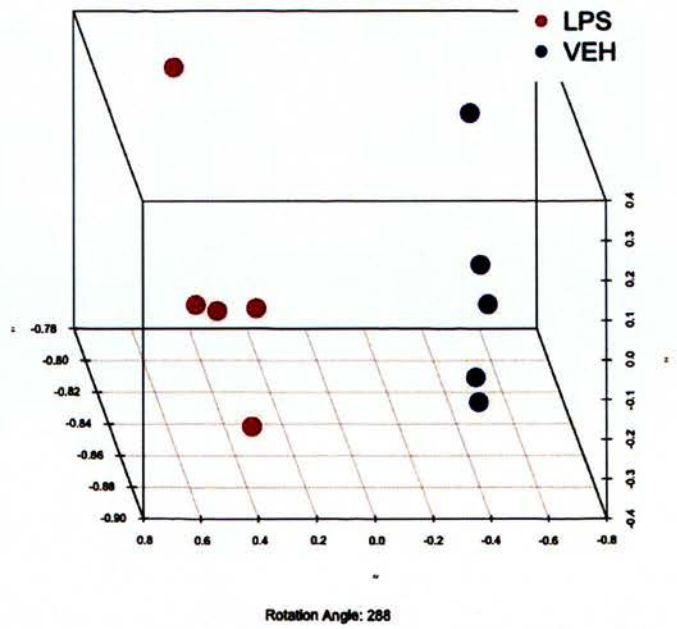


Figure 4.10

PCA plot for comparison of LPS vs Vehicle

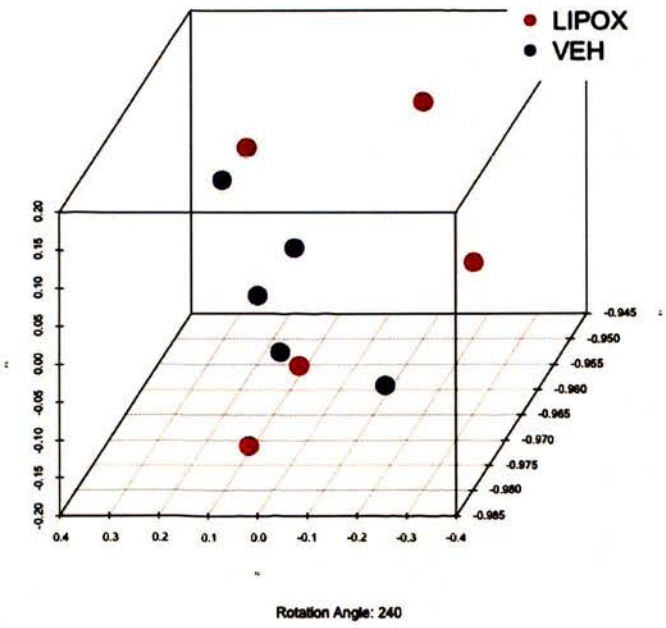


Figure 4.11

PCA plot for comparison of Lipoxin vs Vehicle

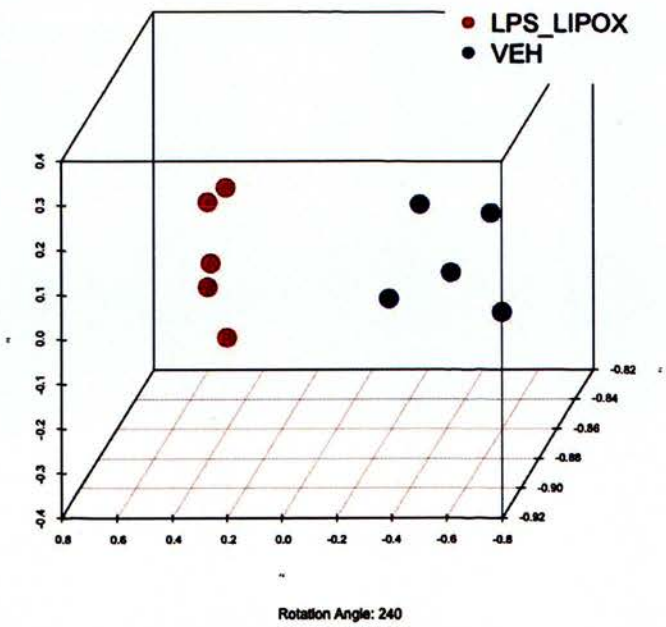


Figure 4.12

PCA plot for comparison of LPS&Lipoxin vs Vehicle

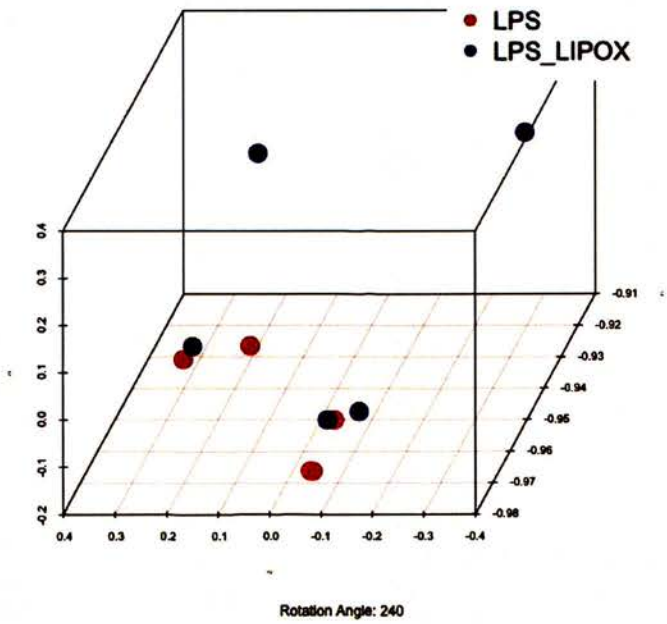


Figure 4.13

PCA plot for comparison of LPS&Lipoxin vs LPS

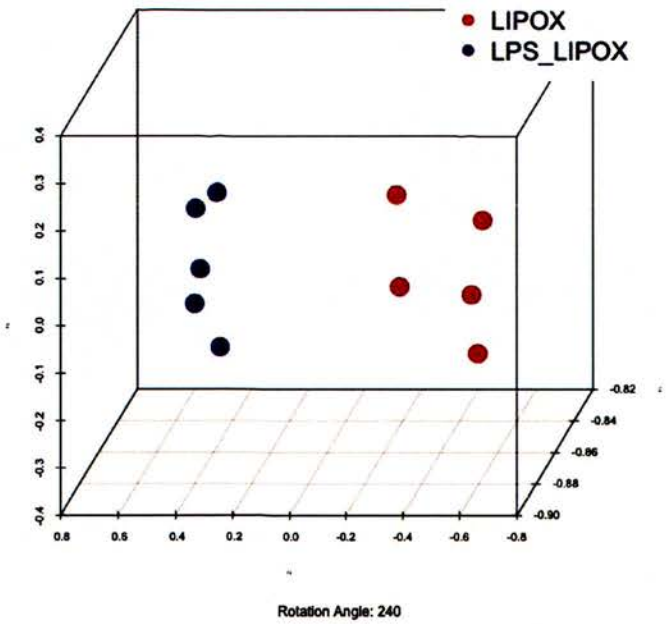


Figure 4.14

PCA plot for comparison LPS&Lipoxin vs Lipoxin

Figs 4.10 to 4.14 illustrate PCA analysis for each of the group comparisons, allowing sample relationships to be visualised in three-dimensional space. Where the groups being compared differ by LPS treatment in can be seen that the dots draw apart into two distinct clusters. However, where the groups being compared differ by LXA4 treatment the dots do not draw into clusters, suggesting that variability between individual patients within the experiment have greater differences than any differences induced by LXA4.

4.3.7. Heatmaps

The following heatmaps (Figures 4.15 to 4.19) provide a graphical representation of all the differentially expressed genes in each of the group comparisons. Each column represents an individual sample, and each row represents a differentially expressed feature, with red indicating upregulation and green representing downregulation. In comparisons where LPS treatment is the group difference, it can be seen that the samples tend to cluster by treatment groups (ie LPS treated or non-LPS treated) and that the columns are fairly similar and tend towards more genes being upregulated rather than downregulated, demonstrating a significant effect of LPS treatment in differential gene expression. However, in group comparisons where lipoxin is the group difference, the samples have clustered by individual patient as opposed to by treatment group, again suggesting that patient variation is more significant than any variation caused by lipoxin.

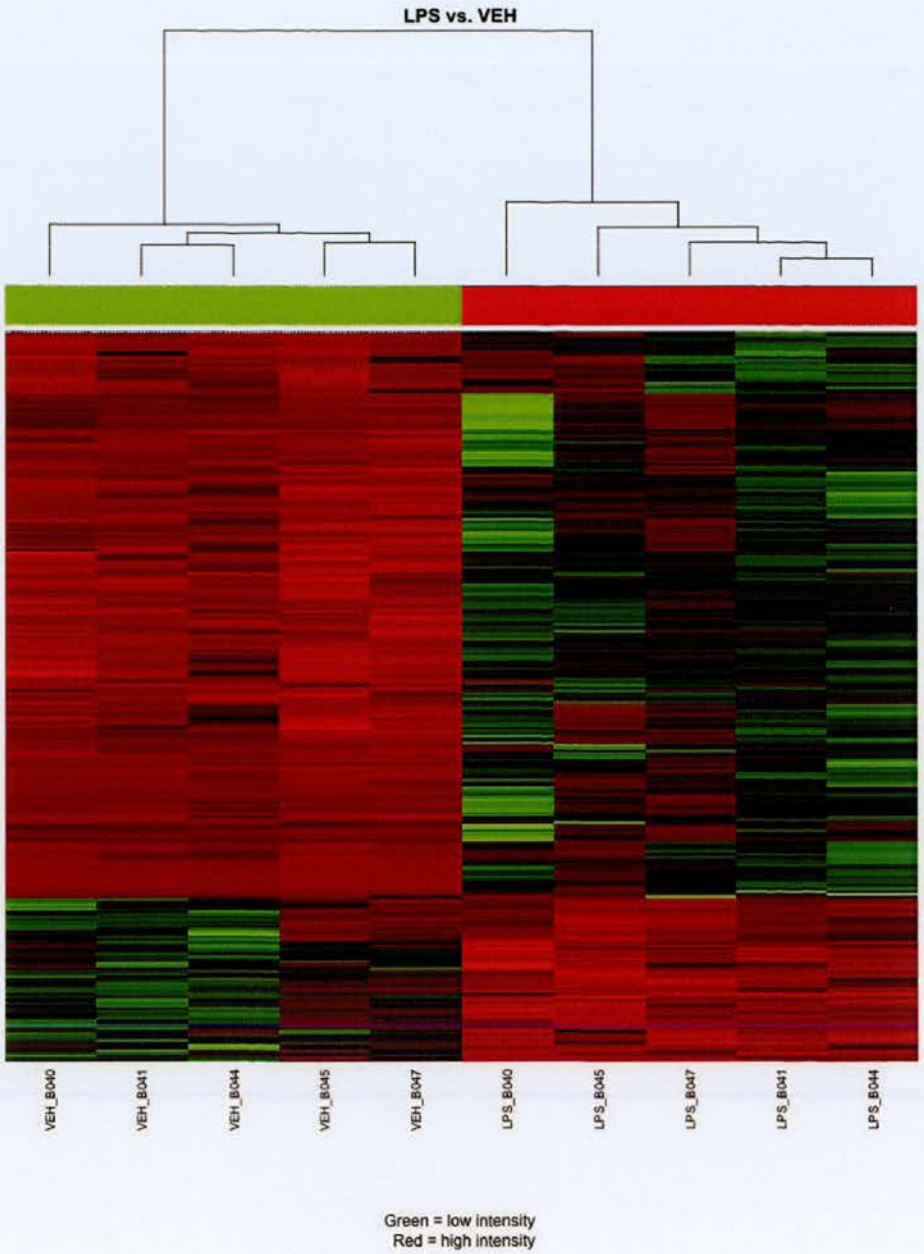


Figure 4.15

HEATMAP plot for comparison of LPS vs Vehicle

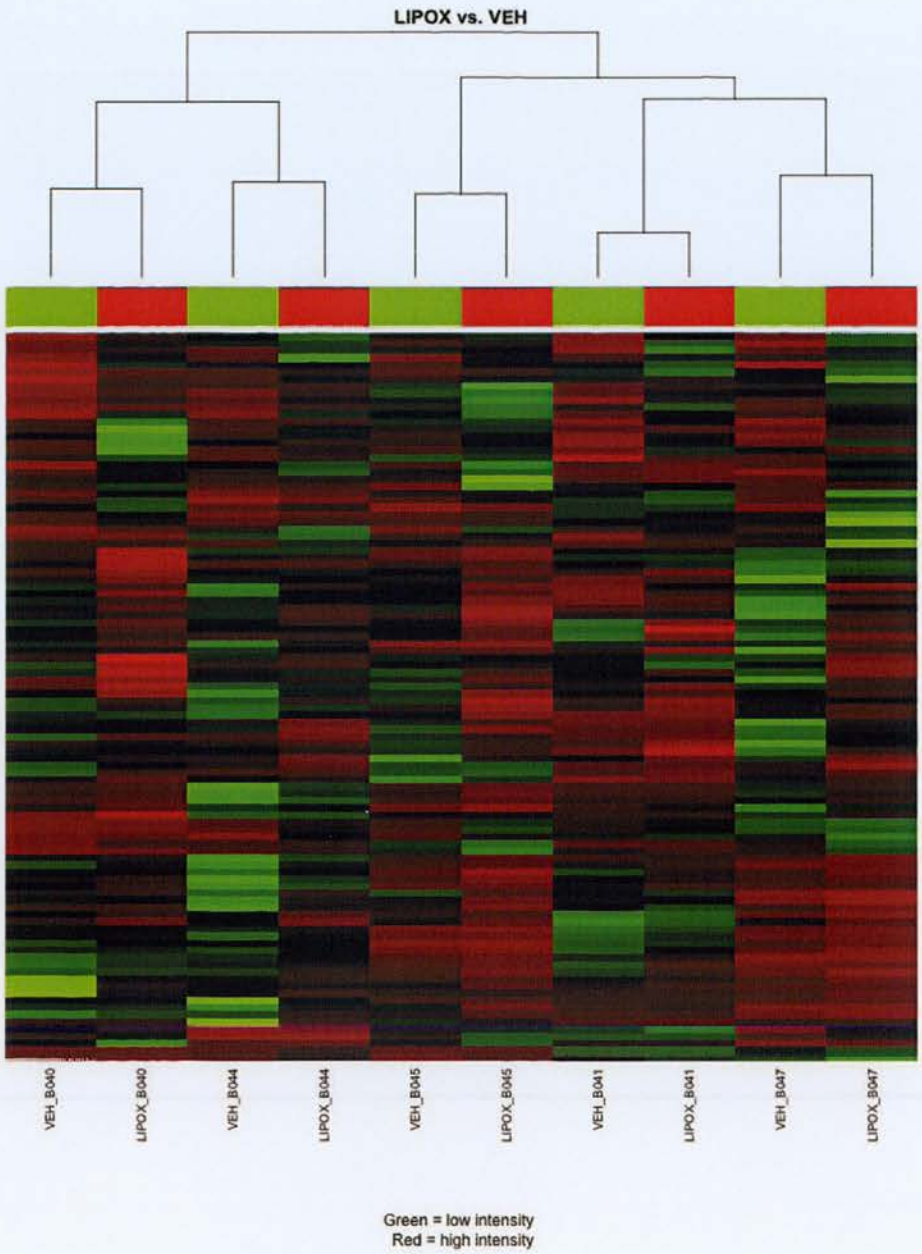


Figure 4.16

HEATMAP plot for comparison of Lipoxin vs Vehicle

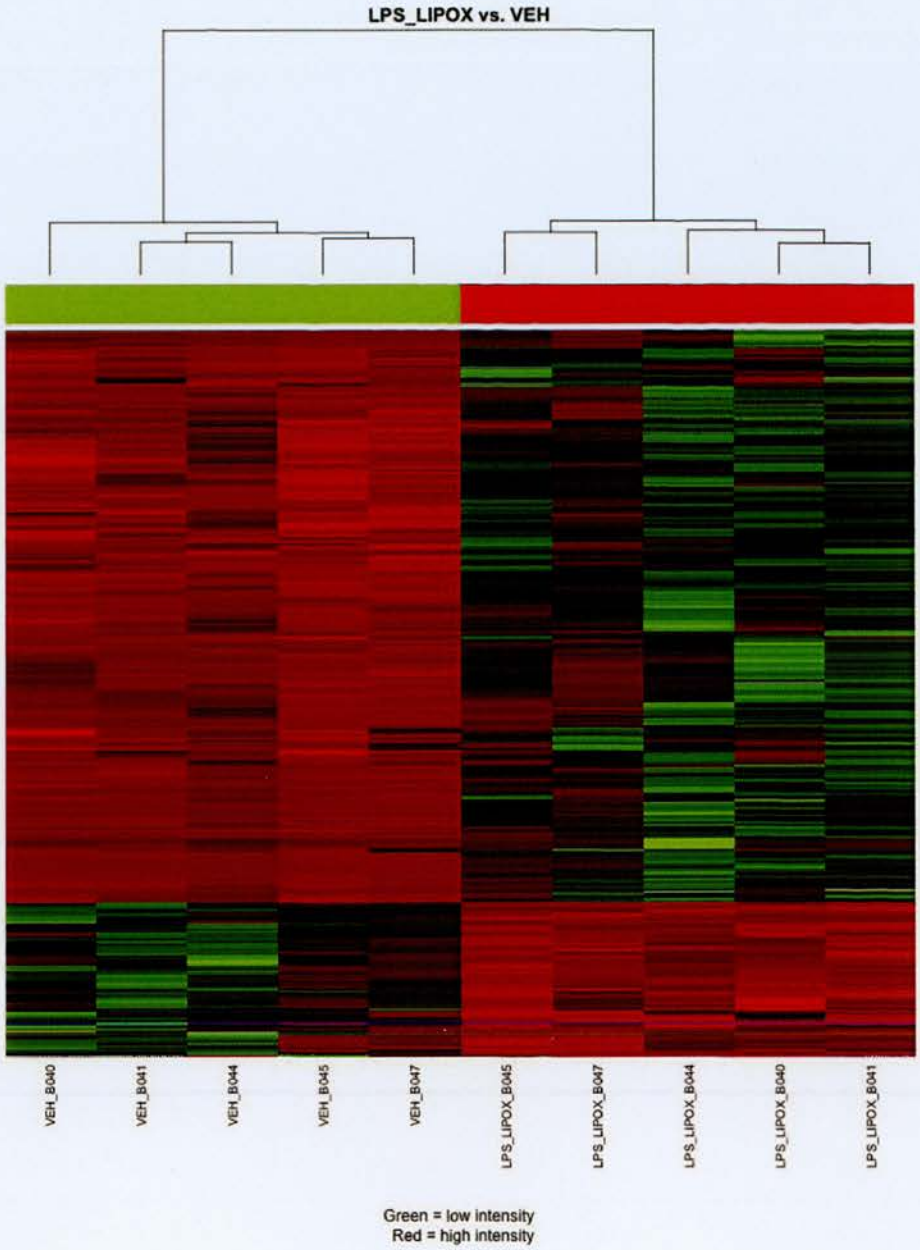


Figure 4.17

HEATMAP plot for comparison of LPS&Lipoxin vs Vehicle

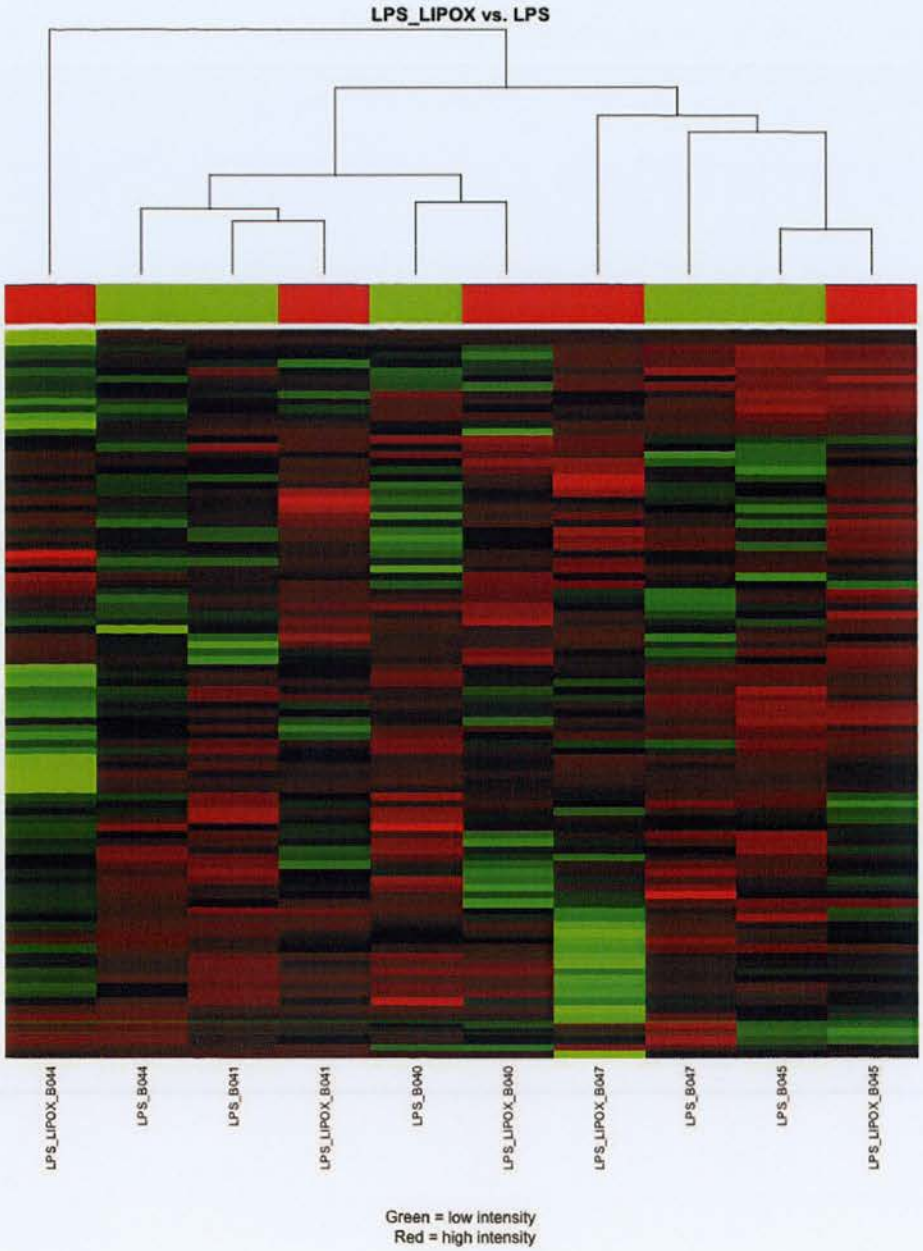


Figure 4.18

HEATMAP plot for comparison of LPS&Lipoxin vs LPS

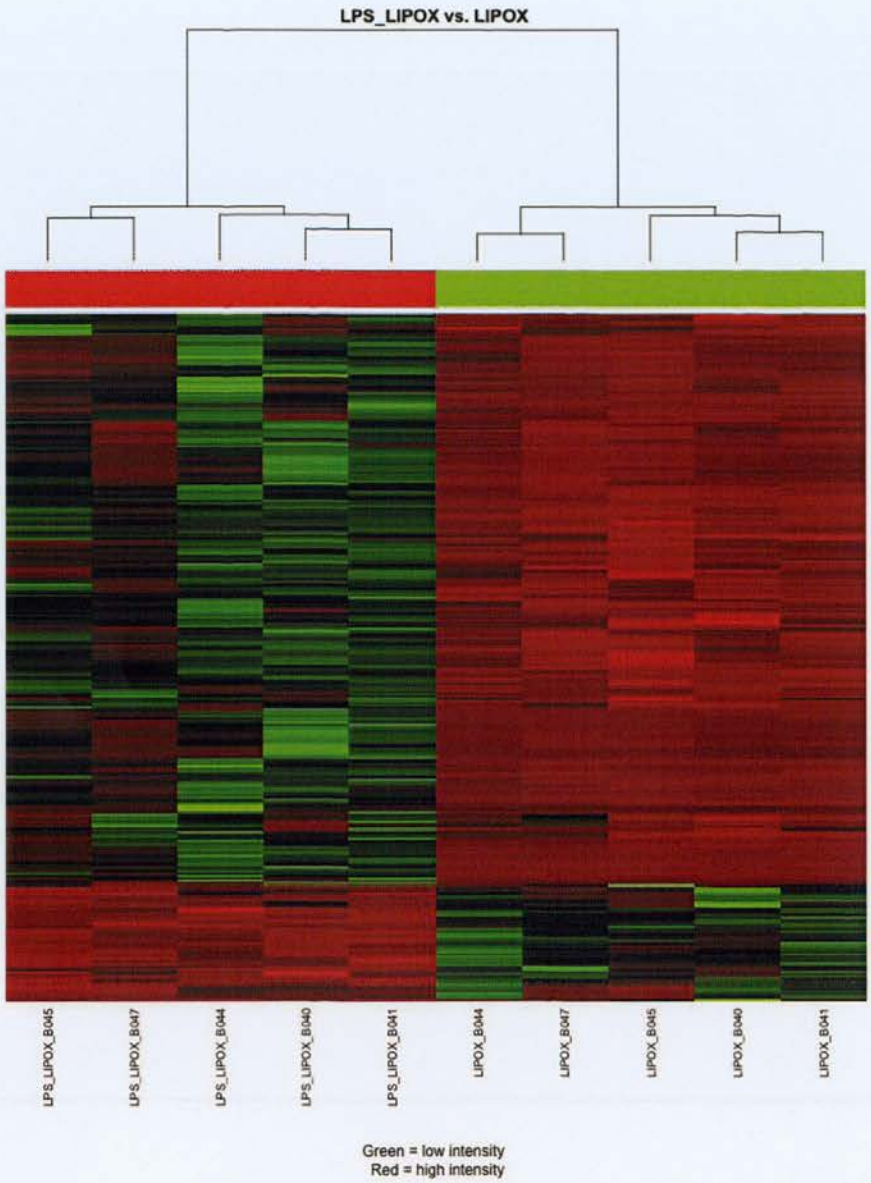


Figure 4.19

HEATMAP plot for comparison LPS&Lipoxin vs Lipoxin

In these heat maps Pearson's metrics is used to cluster the samples and filtered features. The clustering is based on the general expression measurement similarity. In the plots red colour means high expression and green low expression. Each row represents one differentially expressed (DE) feature and each column represents one sample. Again it can be seen that where the groups being compared differ by LPS treatment the samples cluster into treatment groups, whereas where the groups being compared differ by LXA4 treatment the samples tend to cluster by individual.

Figures 4.5 to 4.19 demonstrate that treatment with LPS induced significant changes in the gene profiles but that LXA4 treatment did not. Fold change thresholds had been set for the LXA4 treatment at 1.2 but the analysis demonstrates that there is greater variation between the five individuals than was induced by LXA4 treatment. This is further discussed in the Discussion section of this chapter. The decision was made therefore to concentrate on the analysis of the differentially expressed genes induced by LPS treatment when compared to Vehicle.

4.3.8. Differentially expressed genes LPS vs Vehicle

In the comparison between LPS vs Vehicle, 294 genes were differentially expressed. 228 genes were upregulated and 66 were downregulated. The top 50 differentially expressed probes upregulated and downregulated by treatment with LPS are listed in Tables 4.6 and 4.7. Full lists are found in Appendix 3.

Table 4.6

Top 50 differentially expressed upregulated probes in the comparison of LPS vs Vehicle. The probes are organised by average ranking based on both p-value and fold change.

Symbol	Gene name	Fold change	FDR corrected P-value
SELE	E selectin	53.07	2.85E-13
CCL3	chemokine (C-C motif) ligand 3	27.07	3.51E-13
CX3CL1	chemokine (C-X3-C motif) ligand 1	24.93	5.89E-13
CCL4L2	chemokine (C-C motif) ligand 4-like 2	21.19	1.81E-14
CCL3L1	chemokine (C-C motif) ligand 3-like 1	23.16	4.46E-12
LTB	lymphotoxin beta (TNF superfamily, member 3)	19.27	6.45E-13
CCL3L1	chemokine (C-C motif) ligand 3-like 1	19.23	4.76E-12
CCL3L3	chemokine (C-C motif) ligand 3-like 3	22.76	6.72E-11
IL1A	interleukin 1, alpha	18.35	7.21E-12
IL1B	interleukin 1, beta	42.50	5.96E-10
CCL20	chemokine (C-C motif) ligand 20	23.13	5.38E-10
TRAF1	TNF receptor-associated factor 1	7.79	1.18E-12
CSF3	colony stimulating factor 3 (granulocyte)	13.57	2.02E-10

Symbol	Gene name	Fold change	FDR corrected P-value
TNFAIP2	tumor necrosis factor, alpha-induced protein 2	10.75	1.43E-10
CCL4L1	chemokine (C-C motif) ligand 4-like 1	10.01	1.07E-10
IL23A	interleukin 23, alpha subunit p19	22.73	6.81E-09
LTB	lymphotoxin beta (TNF superfamily, member 3)	6.93	8.49E-10
CCL8	chemokine (C-C motif) ligand 8	9.46	6.98E-09
GCH1	GTP cyclohydrolase 1	6.87	5.54E-09
ADORA2A	adenosine A2a receptor	5.07	3.64E-10
SLC2A6	solute carrier family 2 (facilitated glucose transporter), member 6	4.92	2.02E-10
C15orf48	chromosome 15 open reading frame 48	5.85	4.20E-09
CSF3	colony stimulating factor 3 (granulocyte)	17.63	2.51E-08
IL1RN	interleukin 1 receptor antagonist	6.78	7.98E-09
TNFRSF4	tumor necrosis factor receptor superfamily, member 4	4.95	8.38E-10
CCL3L1	chemokine (C-C motif) ligand 3-like 1	9.73	2.62E-08
CXCL2	chemokine (C-X-C motif) ligand 2	10.12	4.59E-08
TNIP1	TNFAIP3 interacting protein 1	3.94	2.30E-11
UBD	ubiquitin D	12.39	5.35E-08
CFB	complement factor B	6.38	2.24E-08
LIPG	lipase, endothelial	6.35	2.44E-08
LIPG	lipase, endothelial	4.44	7.06E-09
IL7R	interleukin 7 receptor	5.26	3.23E-08
CSF2	colony stimulating factor 2 (granulocyte-macrophage)	9.58	1.50E-07
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	3.42	2.94E-11
VCAM1	vascular cell adhesion molecule 1	8.90	1.39E-07
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	3.18	2.75E-13
G0S2	G0/G1switch 2	6.41	6.50E-08
CITED4	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4	4.78	4.58E-08
GPR84	G protein-coupled receptor 84	4.49	4.58E-08
IL32	interleukin 32	3.70	5.28E-09
IRAK2	interleukin-1 receptor-associated kinase 2	3.40	2.73E-09
ZC3H12A	zinc finger CCCH-type containing 12A	4.09	3.64E-08
HCK	hemopoietic cell kinase	3.27	8.49E-10
NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	3.66	9.23E-09

Symbol	Gene name	Fold change	FDR corrected P-value
MEOX1	mesenchyme homeobox 1	3.73	1.85E-08
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	6.90	4.37E-07
C15orf48	chromosome 15 open reading frame 48	3.77	4.91E-08
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	7.57	6.78E-07
TNFAIP6	tumor necrosis factor, alpha-induced protein 6	4.11	1.82E-07

Table 4.7

Top 50 differentially expressed downregulated probes in the comparison of LPS vs Vehicle. The probes are organised by average ranking based on both p-value and fold change.

Symbol	Gene name	Fold change	FDR corrected P-value
GRRP1	glycine/arginine rich protein 1	-5.72	9.87E-09
PPP1R16B	protein phosphatase 1, regulatory (inhibitor) subunit 16B	-2.79	5.38E-10
INHBB	inhibin, beta B	-3.34	1.38E-07
HAVCR2	hepatitis A virus cellular receptor 2	-2.88	6.02E-08
RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	-2.48	5.85E-11
LHX6	LIM homeobox 6	-2.72	1.92E-07
CARD10	caspase recruitment domain family, member 10	-2.56	3.64E-08
RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	-2.32	7.98E-09
GCHFR	GTP cyclohydrolase I feedback regulator	-2.50	2.55E-07
FPR3	formyl peptide receptor 3	-3.09	2.14E-06
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble	-2.68	1.53E-06
IFI30	interferon, gamma-inducible protein 30	-2.58	1.17E-06
MTUS1	microtubule associated tumor suppressor 1	-2.47	9.92E-07
GAS1	growth arrest-specific 1	-2.55	1.23E-06
GIMAP6	GTPase, IMAP family member 6	-2.28	1.10E-06

Symbol	Gene name	Fold change	FDR corrected P-value
ICAM2	intercellular adhesion molecule 2	-2.46	2.12E-06
MFNG	MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	-2.16	9.29E-07
FUCA1	fucosidase, alpha-L- 1, tissue	-2.67	6.45E-06
C6orf141	chromosome 6 open reading frame 141	-2.26	1.85E-06
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1	-2.71	7.72E-06
KBTBD11	kelch repeat and BTB (POZ) domain containing 11	-2.88	1.09E-05
NOSTRIN	nitric oxide synthase trafficker	-2.03	4.89E-07
MAN1C1	mannosidase, alpha, class 1C, member 1	-2.10	1.34E-06
CPLX1	complexin 1	-2.22	3.26E-06
HSPA12B	heat shock 70kD protein 12B	-2.01	1.10E-06
MALL	mal, T-cell differentiation protein-like	-2.12	3.37E-06
SDS	serine dehydratase	-3.30	3.31E-05
GALM	galactose mutarotase (aldose 1-epimerase)	-2.05	2.75E-06
RNASE6	ribonuclease, RNase A family, k6	-2.60	2.07E-05
NPL	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)	-2.25	9.83E-06
PDE7B	phosphodiesterase 7B	-2.04	3.27E-06
ADAP2	ArfGAP with dual PH domains 2	-2.01	3.31E-06
MBP	myelin basic protein	-2.16	1.21E-05
DACH1	dachshund homolog 1 (Drosophila)	-2.10	1.03E-05
NPL	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)	-2.15	1.59E-05
CD34	CD34 molecule	-2.23	2.62E-05
MTUS1	microtubule associated tumor suppressor 1	-2.09	1.41E-05
MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A	-2.05	1.24E-05
TEK	TEK tyrosine kinase, endothelial	-2.18	2.04E-05
CD34	CD34 molecule	-2.03	1.24E-05
CYTL1	cytokine-like 1	-2.48	4.07E-05
TPD52L1	tumor protein D52-like 1	-2.03	1.44E-05
CYYR1	cysteine/tyrosine-rich 1	-2.15	2.67E-05
LAPTM5	lysosomal protein transmembrane 5	-2.04	1.78E-05
PROX1	prospero homeobox 1	-2.20	3.29E-05
STC2	stanniocalcin 2	-2.05	2.79E-05
VAMP8	vesicle-associated membrane protein 8 (endobrevin)	-2.01	2.79E-05
HLA-DMB	major histocompatibility complex, class II, DM beta	-2.19	5.62E-05
CD300A	CD300a molecule	-2.09	4.07E-05
GRAP	GRB2-related adaptor protein 2	-2.03	3.34E-05

4.3.9. Functional analysis

Functional analysis of the data was provided by uploading the lists of differentially expressed genes into Metacore by GeneGo. This data set is then matched to terms within GeneGo functional ontologies to produce ranked representations of ontologies that are most saturated or 'enriched' with the differentially expressed gene list that was uploaded. GeneGo Process Networks consist of around 110 cellular and molecular processes which have been defined and annotated by GeneGo. Each process represents a network of protein interactions characteristic for the process. The top ten scored Process Networks identified by pathway analysis in the GeneGo database are listed in Figure 4.20.

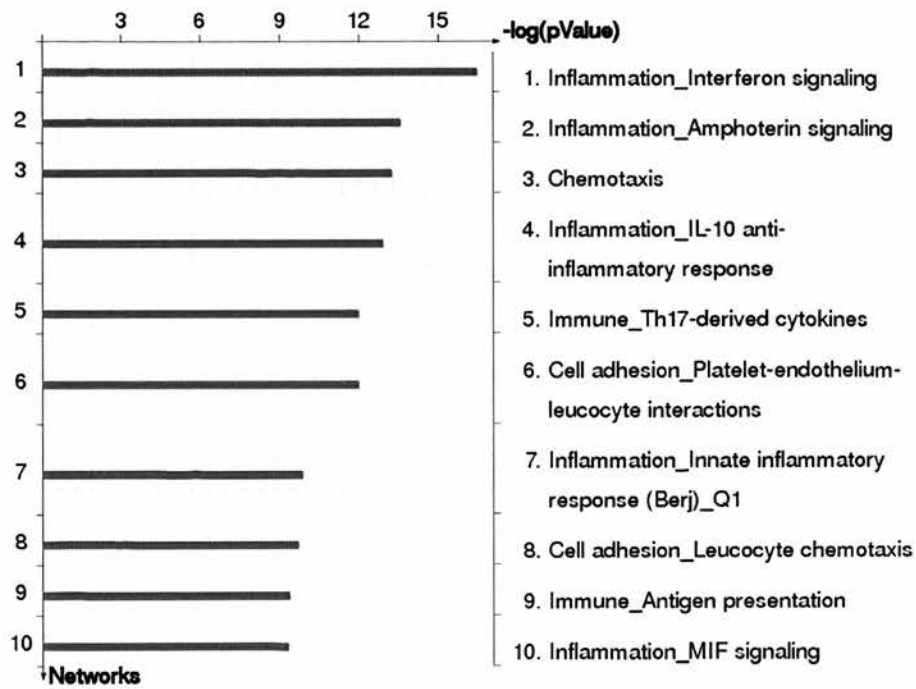


Figure 4.20

Top ten Process Networks for LPS vs Vehicle. The histogram represents process networks that were significantly enriched by the data set of differentially expressed genes in the LPS vs Vehicle experiment. The size of the bar corresponds to –log(pValue) significance. All ten Process Networks are related to inflammation or the immune response.

GeneGo Pathway Maps represent another ontology which consists of a set of about 650 signalling and metabolic maps. They are drawn by GeneGo annotators and manually curated based on confirmed, published protein interactions. The top ten most statistically significant canonical Pathway Maps in the GeneGo database for the experiment LPS vs Vehicle are shown in Figure 4.21 and the top scored map is illustrated in Figure 4.22.

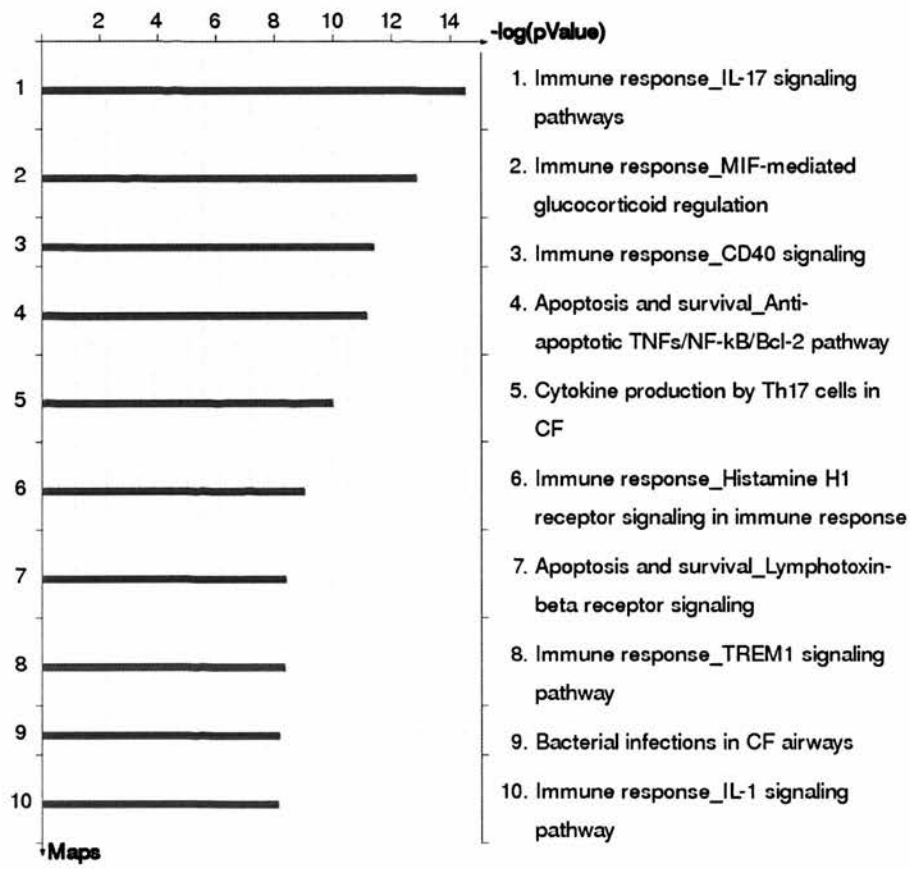


Figure 4.21

Top ten most statistically significant GeneGo Pathway Maps for LPS vs Vehicle. The histogram represents GeneGo Pathway Maps that were significantly enriched by the data set of differentially expressed genes in the LPS vs Vehicle experiment. The height of the bar corresponds to –log(pValue) significance.

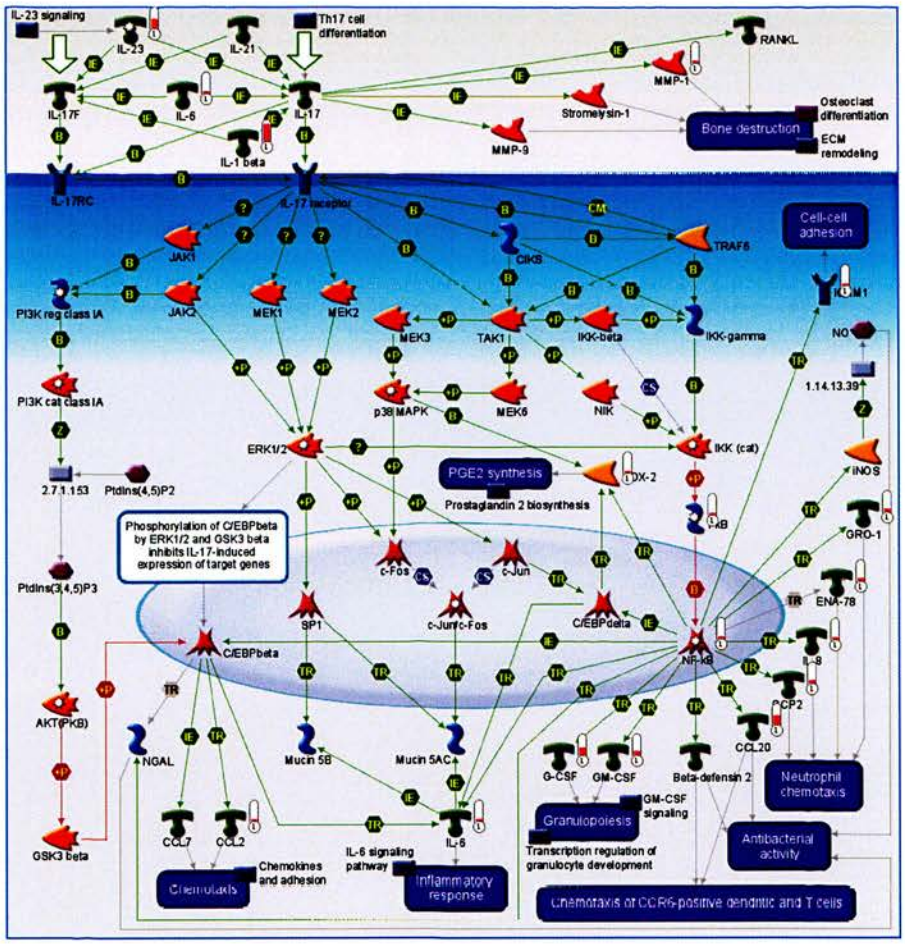


Figure 4.22 Top scored map

The interleukin-17 signalling pathway (GeneGo) Display of differentially expressed genes in human myometrial explants cultures with LPS vs Vehicle mapped on the interleukin-17 signalling pathway. Red thermometers indicate upregulation of that gene with LPS treatment. Of note is the area in the bottom right of the diagram in which an entire network module is upregulated by LPS.

4.3.10. Validation by RT-PCR

I was interested in looking at pathways which might be common to the stimulation of myometrium by LPS and the changes that may be observed in labouring vs non-labouring tissue. A recently published paper by Bollopragada et al looked at differentially expressed genes when mRNA from cervical and lower uterine segment myometrial biopsies from labouring and non-labouring women were compared (Bollopragada et al. 2009). This study identified 110 genes that were upregulated in

both myometrial cervical biopsies of labouring women when compared with non-labouring. This list of differentially expressed genes was compared with the differentially expressed upregulated genes from our experiment of LPS vs Vehicle. 19 genes were common to both lists and are listed in Table 4.8. From this list 5 genes were selected and the results validated using quantitative RT-PCR.

Symbol	Gene name	Fold change	FDR corrected P-value
SELE	**Selectin E**	**2.84858E-13**	**53.067**
CSF3	**Colony stimulating factor 3 (granulocyte)**	**2.01708E-10**	**13.566**
CCL20	Chemokine (C-C motif) ligand 20	5.37874E-10	23.128
IL1B	Interleukin 1, beta	5.96163E-10	42.496
LIPG	Lipase, endothelial	7.05514E-09	4.437
CXCL2	Chemokine (C-X-C motif) ligand 2	4.58772E-08	10.115
PDE4B	Phosphodiesterase 4B, cAMP-specific	2.30756E-07	2.611
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	4.37033E-07	6.903
ICAM1	**Intercellular adhesion molecule 1**	**1.0189E-06**	**2.594**
MMP1	**Matrix metallopeptidase 1**	**1.41306E-06**	**2.428**
SLC25A37	Solute carrier family 25, member 37	3.11364E-06	2.77
CCL2	**Chemokine (C-C motif) ligand 2**	**3.77367E-06**	**3.487**
AQP9	Aquaporin 9	9.65526E-06	2.683
LIF	Leukemia inhibitory factor (cholinergic differentiation factor)	1.32622E-05	3.728
CXCL5	Chemokine (C-X-C motif) ligand 5	1.66431E-05	4.998
TREM1	Triggering receptor expressed on myeloid cells 1	2.37638E-05	2.155
IL8	Interleukin 8	8.57917E-05	3.864
IL6	Interleukin 6	0.00021575	4.121
PTGS2	Prostaglandin-endoperoxide synthase 2	0.000511241	3.313

Table 4.8

Differentially expressed upregulated genes common to LPS vs Vehicle and an array comparing labouring vs non-labouring myometrium and cervix (Bollopragada et al. 2009). Fold changes and p-values are those from the LPS vs Vehicle comparison. The genes selected for validation are highlighted in bold.

For each of the five selected genes, relative mRNA expression was greater in the LPS and LPS&Lipoxin groups than in the Vehicle or Lipoxin groups (Figures 4.23, 4.25, 4.27, 4.29 and 4.31). This was consistent with the findings of the microarray, validating its results.

When labouring and non-labouring samples were compared, significantly greater relative mRNA expression was noted in E-selectin, CSF3, ICAM1 and CCL2 (Figures 4.24, 4.26, 4.28 and 4.32).

Relative mRNA expression of MMP1 appeared to be greater in labouring tissue but this was not statistically significant (Figure 4.30). Expression levels were extremely low in all the non-labouring samples, and in some of the labouring samples, but were very high in others of the labouring samples. This wide variability accounts for the appearance of a greater expression in labouring tissue, but not a statistically significant result.

E Selectin

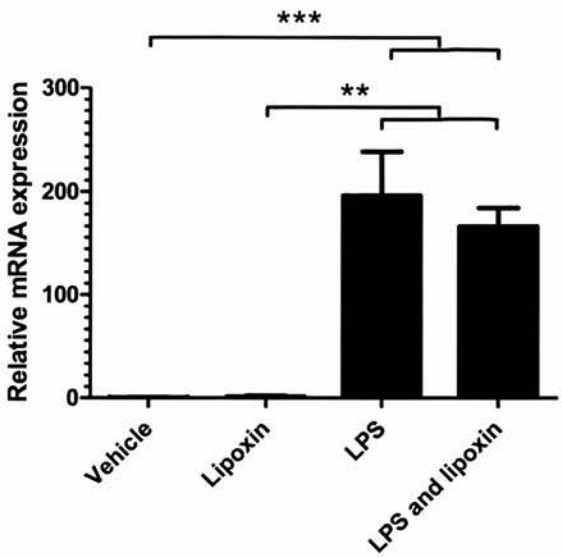


Figure 4.23 E-selectin mRNA expression in myometrial explants
Relative mRNA expression of E-selectin in term myometrial explants (n=5) cultured for 8 hours with vehicle, lipoxin A4 100nM, LPS 100ng/ml or lipoxin A4 and LPS together. Expression was significantly greater in explants treated with either LPS or LPS & lipoxin A4 when compared to either vehicle or lipoxin A4 alone. Lipoxin A4 alone had no effect when compared to vehicle. **p< 0.01, ***p<0.001; ANOVA and Bonferroni's post test.

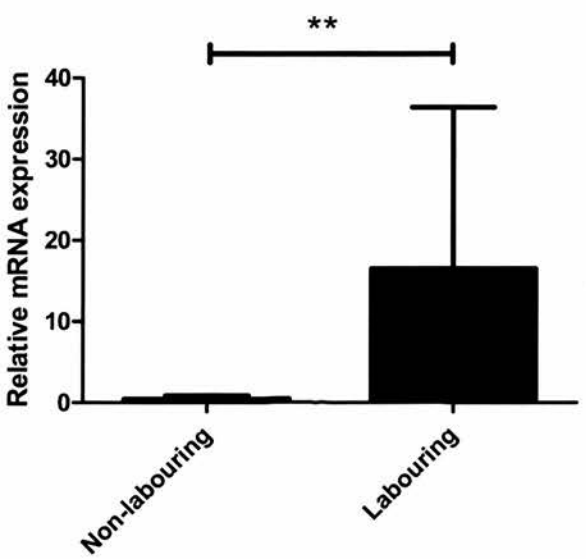


Figure 4.24 E-selectin mRNA expression in labouring and non-labouring myometrium
Relative mRNA expression of E-selectin is greater in labouring term myometrium than in non-labouring. Data are presented as median and inter-quartile range; n=11 in each group; ** p=0.0058, Mann-Whitney test.

Colony stimulating factor 3 (CSF3)

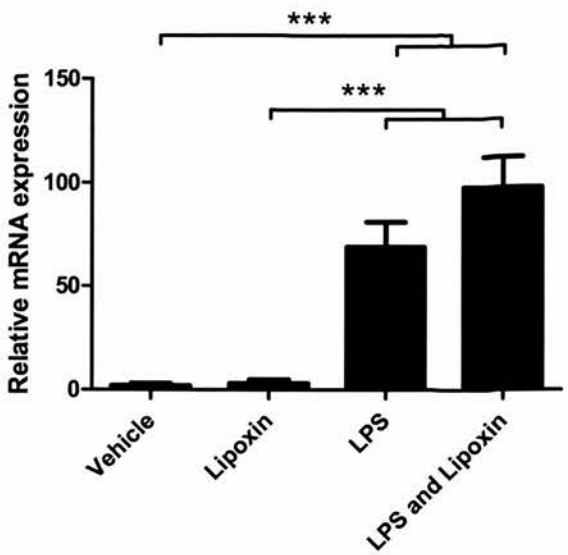


Figure 4.25 CSF3 mRNA expression in myometrial explants
Relative mRNA expression of CSF3 in term myometrial explants (n=5) cultured for 8 hours with vehicle, lipoxin A4 100nM, LPS 100ng/ml or lipoxin A4 and LPS together. Expression was significantly greater in explants treated with either LPS or LPS & lipoxin A4 when compared to either vehicle or LXA4 alone. LXA4 alone had no effect when compared to vehicle. ***p<0.001; ANOVA and Bonferroni's post test.

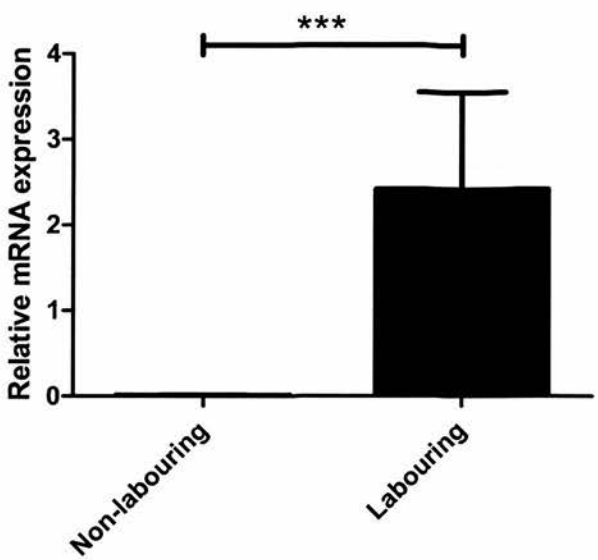


Figure 4.26 CSF3 mRNA expression in labouring and non-labouring myometrium
Relative mRNA expression of CSF3 is greater in labouring term myometrium than in non-labouring. Data are presented as median and interquartile range; n=11 in each group; ** p=0.0006, Mann-Whitney test.

Intercellular adhesion molecule 1 (ICAM1)

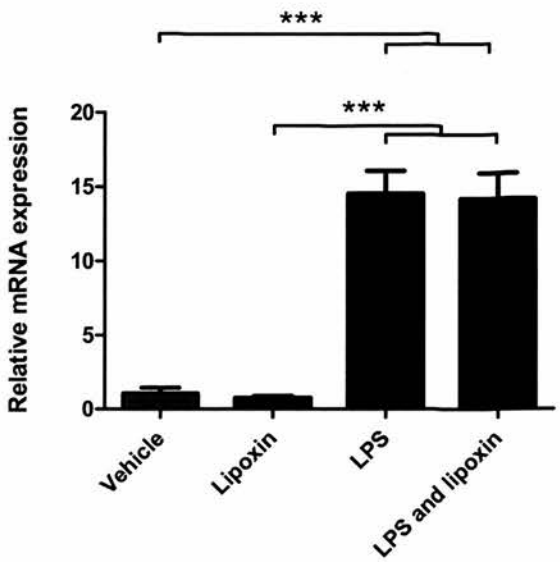


Figure 4.27 ICAM1 mRNA expression in myometrial explants
Relative mRNA expression of ICAM1 in term myometrial explants (n=5) cultured for 8 hours with vehicle, lipoxin A4 100nM, LPS 100ng/ml or lipoxin A4 and LPS together. Expression was significantly greater in explants treated with either LPS or LPS & lipoxin A4 when compared to either vehicle or LXA4 alone. LXA4 alone had no effect when compared to vehicle. ***p<0.001; ANOVA and Bonferroni's post test.

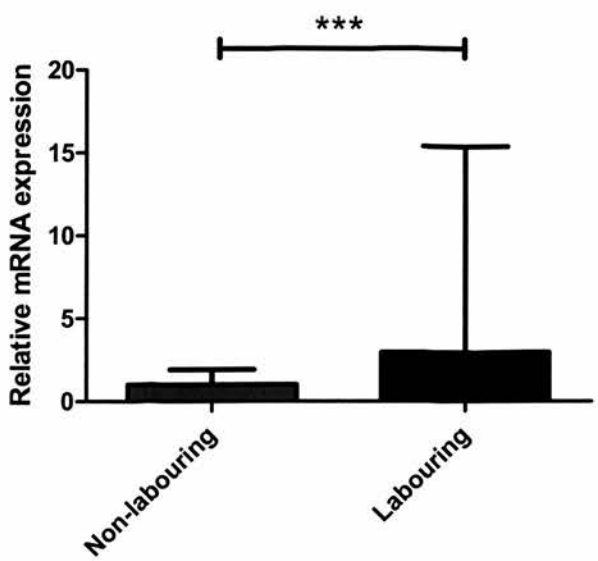


Figure 4.28 ICAM1 mRNA expression in labouring and non-labouring myometrium
Relative mRNA expression of ICAM1 is greater in labouring term myometrium than in non-labouring. Data are presented as median and interquartile range; n=11 in each group; ** p=0.0004, Mann-Whitney test.

Matrix metallopeptidase 1 (MMP1)

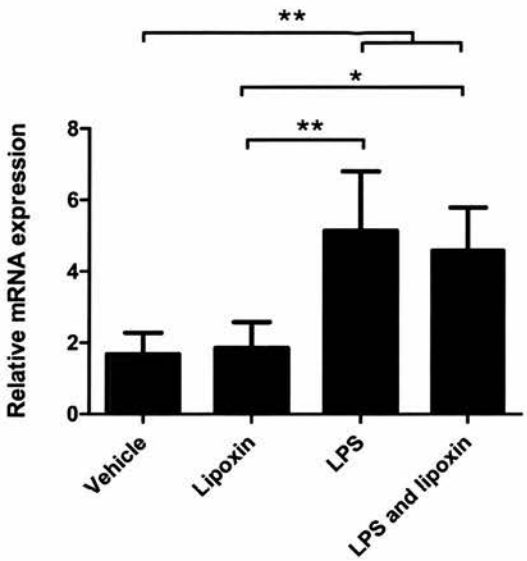


Figure 4.29 MMP1 mRNA expression in myometrial explants
Relative mRNA expression of MMP1 in term myometrial explants (n=5) cultured for 8 hours with vehicle, lipoxin A4 100nM, LPS 100ng/ml or lipoxin A4 and LPS together. Expression was significantly greater in explants treated with either LPS or LPS & lipoxin A4 when compared to either vehicle or lipoxin alone. Lipoxin alone had no effect when compared to vehicle. *p<0.05, **p< 0.01; ANOVA and Bonferroni's post test.

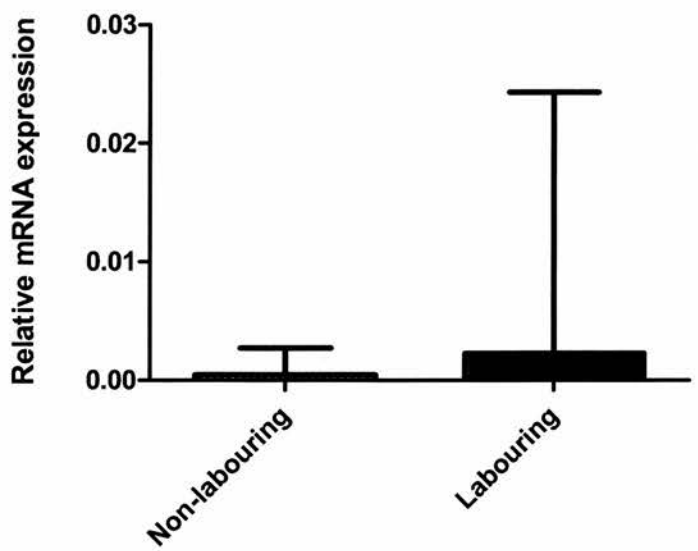


Figure 4.30 MMP1 mRNA expression in labouring and non-labouring myometrium
Relative mRNA expression of MMP1 is not significantly different in labouring term myometrium than in non-labouring. Data are presented as median and interquartile range; n=11 in each group; p=0.21, Mann-Whitney test.

Chemokine (C-C motif) ligand 2 (CCL2)

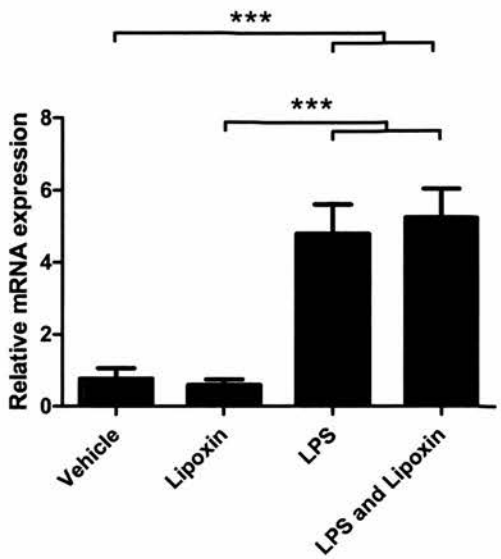


Figure 4.31 CCL2 mRNA expression in myometrial explants
Relative mRNA expression of CCL2 in term myometrial explants (n=5) cultured for 8 hours with vehicle, lipoxin A4 100nM, LPS 100ng/ml or lipoxin A4 and LPS together. Expression was significantly greater in explants treated with either LPS or LPS & lipoxin A4 when compared to either vehicle or lipoxin alone. Lipoxin alone had no effect when compared to vehicle. ***p<0.001; ANOVA and Bonferroni's post test.

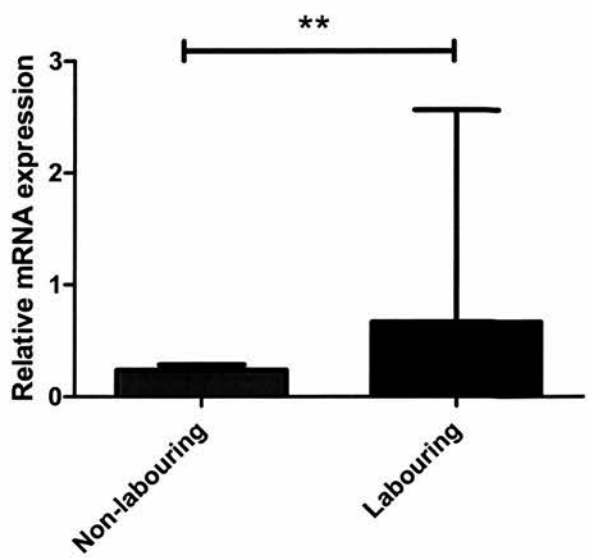


Figure 4.32 CCL2 mRNA expression in labouring and non-labouring myometrium
Relative mRNA expression of CCL2 is greater in labouring term myometrium than in non-labouring. Data are presented as median and interquartile range; n=11 in each group; ** p=0.025, Mann-Whitney test.

4.4. DISCUSSION

The original design of this experiment aimed to use functional genomics to explore the anti-inflammatory or pro-resolution functional effects that LXA4 may have on inflammatory processes within human pregnant myometrium, as has been observed and published by our group (Maldonado-Perez et al. 2010). Unfortunately, the results were not as exciting as had been hoped, because the fold changes in differentially expressed genes in the groups treated with LXA4 were so small as to have demonstrated no significant effect of LXA4. Subsequently it was discovered that other groups had also found they had been unable to reproduce results with LXA4 from the same manufacturer, and that spectral analysis demonstrated contamination of the LXA4 (personal communication Prof C Godson, University College Dublin). It appears that this contamination of LXA4 may have rendered it inactive. Thus, it is not possible to draw any conclusions from this experiment on how LXA4 may be having anti-inflammatory or pro-resolution effects by altering gene expression in myometrium. However, the microarray results did show profound effects in gene expression by treating with LPS. These results are of value in identifying inflammatory networks existing in myometrium, comparing them with the inflammatory effects of parturition and exploring how these comparisons may be used in the future to find new ways of manipulating abnormal inflammation in myometrium.

LPS is pro-inflammatory and is widely used in research to mimic the inflammatory process of labour and pre-term labour, and to induce preterm labour in animal models (Fidel et al. 1994; Elovitz et al. 2003). This approach provides a useful model for term and pre-term labour, but of course the conclusions that may be drawn from such experimental models are limited by the fact that pre-term and term labour involve complex, multi-faceted pathways, not just those involving inflammation.

Whilst there is a great deal published on the inflammatory effect of LPS on human pregnant myometrium, to the best of my knowledge, this is the first time that a genomics approach has been used to explore the pathways by which LPS exerts its

effects in myometrium, and then compares this to known pathways which have been demonstrated in labour. As would be expected from LPS, I found that the top processes were related to inflammation, including chemotaxis and cell adhesion processes. This is consistent with the published data of the effects of LPS, both in myometrium (Sehringer et al. 2000; Diamond et al. 2007), and in other systems of the body including the respiratory system (Bannerman and Goldblum 2003), gastrointestinal tract (Petersson et al. 2011) and brain (Burd et al. 2010). Excitingly, when the differentially expressed genes, top-scoring processes and maps were compared with published data on labouring versus non-labouring comparisons, it was possible to observe some very interesting comparisons.

For example, when the list of differentially expressed genes generated by treating myometrium with LPS was compared with the list of differentially expressed genes in labouring vs non-labouring myometrium and cervix from the work published by Bollopragada et al (Bollopragada et al. 2009), 19 common genes were identified. It was from this list that 5 genes were chosen to validate the array using quantitative RT-PCR and indeed it was demonstrated that relative mRNA expression was increased in both myometrial samples treated with LPS, and in labouring myometrial samples when compared with non-labouring.

Another recently published functional genomics study of labouring and non-labouring myometrium reported that the myometrium of term labouring women is characterised by upregulation of genes of the inflammatory response and leukocyte chemotaxis (Mittal et al. 2010). In this study the authors also utilised Metacore by GeneGo to analyse their data and it is interesting to note that the upregulated genes in their data set also significantly enriched the interleukin-17 signalling pathway, which was the most highly enriched map by the data from the LPS vs Vehicle experiment presented in this chapter. Indeed, representation of the genes featured on their published map is remarkably similar to the upregulated genes represented on the map illustrated in this chapter in Figure 4.22.

Thus, it may be concluded that there are a number of differentially expressed genes and molecular processes that are common to both parturition and artificial

stimulation of inflammation with LPS, providing valuable evidence that using LPS in animal and explant modelling of parturition can be valid and has great potential for elucidating new avenues of investigation.

4.4.1. Genes chosen for validation

Five genes were chosen from the list of nineteen that were common to the lists of differentially expressed genes from the LPS vs Vehicle comparison and the genes upregulated in labour in Bollopragada et al's analysis (Bollopragada et al. 2009).

E-selectin and ICAM-1 are both leucocyte adhesion molecules and play an important part in recruiting leucocytes to the site of inflammation (Boyd et al. 1988; Bevilacqua et al. 1989). Both E-selectin and ICAM-1 expression are widely reported to be upregulated by LPS via NF-κB activation (Montgomery et al. 1991; Chen et al. 1995; Jersmann et al. 2001) and so the findings of this microarray and subsequent validation are consistent with this. One of the main anti-inflammatory effects of LXA4 is to reduce leucocyte trafficking via an inhibitory effect on leucocyte adhesion molecules (Papayianni et al. 1996; Filep et al. 1999). LXA4 stable analogs have been reported to reduce the over-expression of E-selectin brought about by LPS in HUVEC cells (Fiorucci et al. 2003) and by IL-1β in endothelial cells (Nascimento-Silva et al. 2005), as well as inhibiting ICAM-1 expression via an NF-κB-dependent mechanism (Decker et al. 2009).

CCL2 is a cytokine also known as monocyte chemotactic protein-1 (MCP-1) that recruits inflammatory cells to sites of inflammation and is involved in macrophage activation. It has been shown to be increased in the myometrium (Esplin et al. 2005), fetal membranes (Esplin et al. 2005) and amniotic fluid (Esplin et al. 2003; Esplin et al. 2005) of term labouring women and has also been shown to be involved in LPS-induced preterm labour in the mouse (Diamond et al. 2007). In rats, CCL2 integrates mechanical and endocrine signals involved in uterine inflammation and parturition and has been suggested as a potential therapeutic target for preterm labour (Shynlova et al. 2008). Validation of the results from this array confirmed that CCL2 was upregulated by both LPS and labour in parturition. LXA4 attenuates TNF-α

stimulated CCL2 release in colonic mucosal cells (Goh et al. 2001) whereas CCL2 expression is increased by serum amyloid A, a pro-inflammatory mediator signalling through the FPR2/ALX receptor (Lee et al. 2008). CCL2 and LXA4-FPR2/ALX interactions may therefore be another interesting starting point for investigation of the role of LXA4 in parturition.

CSF3 is a cytokine, also known as granulocyte colony-stimulating factor (G-CSF) that stimulates granulocyte production and function (Welte et al. 1985 519). It also has an effect on neutrophil function, promoting their survival, proliferation and function (Begley et al. 1986; Lopez et al. 1986). Its production is stimulated by LPS (Vellenga et al. 1988; Dower et al. 2008). Recombinant G-CSF is used clinically within oncology, to treat patients receiving chemotherapy, as it counteracts chemotherapy-induced neutropenia (Morstyn et al. 1989; Sheridan et al. 1989). G-CSF has been used in the treatment of septic preterm neonates (Kucukoduk et al. 2002), although a Cochrane review does not recommend its routine use due to lack of evidence of efficacy (Carr et al. 2003). G-CSF has also been shown to be useful in limiting tissue damage in the inflammatory states of burn injury and myocardial infarction and these effects are possibly achieved by promoting homeostasis as opposed to overstimulation of the immune system (Peter et al. 1999; Baldo et al. 2011). Little is known about its effects in utero-placental tissues or in the process of parturition, or interactions it may have with LXA4. Given that it is upregulated in labour and may have positive effects in limiting damage by an over-active immune response, it may be an interesting target for investigation within the inflammatory process of labour.

MMP1 is a matrix metalloproteinase that, like the other proteins of the same family, is involved in the breakdown of ECM in both normal physiological processes and disease processes (Contasta et al. 1999; Dunleavey et al. 2000). It is involved in the degradation of the ECM of the fetal membranes that lead to rupture of the membranes during/prior to labour and genetic polymorphisms of MMP1 have been associated with an increased risk of PPROM (Maymon et al. 2000; Fujimoto et al. 2002; Wang et al. 2008). MMP1 production has been reported to be stimulated by LPS (La et al. 2009), as the array in this chapter also confirms, and chorioamnionitis

is associated with increased amniotic fluid levels of MMP1 (Maymon et al. 2000), likely through increased cytokine induction (Oner et al. 2008). LXA4 has been shown to decrease LPS-induced production of some pro-inflammatory cytokines (Maldonado-Perez et al. 2007) and so it would be interesting to explore whether or not it can suppress LPS-induced production of MMP1 and other metalloproteinases.

4.4.2. Summary

LXA4 has been demonstrated to have an inhibitory effect on LPS-stimulated production of IL-6 and IL-8 in human pregnant myometrium. These two interleukins featured in the top 30 differentially expressed genes of labouring myometrium in Bollopragada et al's work, and were also upregulated by LPS in the experiment reported in this chapter. LXA4 also has well established pro-resolution and anti-inflammatory effects in other systems of the human body, as detailed in the introduction of this thesis. Further evidence that LXA4 may potentially have an effect in the inflammatory process of labour is demonstrated in the finding that its receptor, FPR2/ALX is upregulated in human parturition. Unfortunately, as previously discussed, due to contamination of the LXA4 used in this experiment, I was unable to confirm any effect of LXA4 on IL-6 or IL-8 in the microarray, or to identify other differentially expressed genes and pathways which may show similar functional effects of LXA4.

Recent work within our laboratory using an alternative source of LXA4 has begun to show results consistent with the previously demonstrated functional effects already published. LPS is successfully used to induce preterm labour in animal models and has, in this work, been shown using functional genomics to share many of the same molecular pathways as parturition itself. If it can be demonstrated that it is possible to manipulate these pathways with pro-resolution mediators, it is possible that in the future, such mediators may be used in the treatment of abnormal or preterm labour. Future experiments therefore may include repeating the work presented in this chapter, using an alternative source of LXA4, or choosing key pathways and differentially expressed genes in the LPS arm of the experiment and examining the effects of LXA4 within these pathways.

5. The effects of hypoxia on the production of Lipoxin A4

5.1. INTRODUCTION

The initiation of parturition and the mechanisms which govern it are as yet, incompletely understood, and the characteristic inflammatory profile that is seen during labour (Thomson et al. 1999) undoubtedly has many potential triggers. Work in the previous chapter explored the myometrial transcriptome in induced inflammation and compared this to the effect seen in labour. Inflammation can lead to tissue hypoxia, for example, as seen clinically in abscess formation. There is also evidence that hypoxia may play a role in promoting inflammatory processes. Hypoxia has been demonstrated to increase expression of key pro-inflammatory mediators IL-6 (Bowen et al. 2005), IL-8 (Karakurum et al. 1994; Bowen et al. 2005), IL-1β (Malek et al. 2001) and PGE_2 (Supramaniam et al. 2004) and there is also a placental increase noted in such mediators in conditions of pregnancy that are associated with an inflammatory state such as placental insufficiency (Wang et al. 2003) and chorioamnionitis (Griesinger et al. 2001). Thus it would appear that relative hypoxia may potentially be one of the features involved in the onset or continuation of the inflammatory process of labour.

Whilst hypoxia may have a role to play in amplifying the inflammatory environment necessary for labour, hypoxia within the utero-placental unit can of course be severely detrimental, both to the process of effective labour, and to the fetus, who until delivery is entirely dependent upon oxygenation from the maternal circulation to survive. During labour, regular uterine contractions episodically constrict the blood vessels that supply the myometrium with oxygen (Borell et al. 1964). Blood vessel constriction appears to be worse when contractions are augmented with oxytocin (Li et al. 2003). Uterine contractions may be sufficient to create intermittent periods of relative hypoxia, both for the myometrial smooth muscle and for the fetus (Peebles et al. 1994). Hypoxia inhibits myometrial smooth muscle contractions in vitro in the human (Bugg et al. 2006) and in the rat (Rhee et al. 1996). In vivo, women undergoing caesarean section for dysfunctional labour have reduced pH of the myometrial capillary blood compared to women in normal labour or women not in labour (Quenby et al. 2004). The importance of dysfunctional labour cannot be

underestimated as it is a common indicator for emergency caesarean section (Thomas et al. 2000), with all of its attendant implications for maternal morbidity and mortality (Lyons 2008). Thus, understanding the contribution of hypoxia to ineffective myometrial contractions is important.

Clearly therefore, a paradox exists in that hypoxia could contribute to the process of parturition by promoting inflammation, but could also be a detrimental feature, by making uterine activity and thus labour itself less effective and also potentially contributing to a damaging environment for the fetus. The effects of hypoxia on several key pro-inflammatory mediators are noted above. The lipid mediator LXA4 has anti-inflammatory and pro-resolution properties, and in myometrium has been demonstrated to reduce LPS-induced production of IL-6 and IL-8 (Maldonado-Perez et al. 2010). However, the effect of hypoxia on anti-inflammatory and pro-resolution mediators and in particular on LXA4 has not been studied, and has not been looked at within the context of parturition.

Aims

The work in this chapter aims:

- to explore how hypoxia may affect the production of LXA4 by myometrial tissue
- to examine the effect of hypoxia on ALOX5 and FPR2/ALX expression in myometrium

A myometrial explant experiment was designed in which tissue was cultured in different oxygen conditions and treated with either vehicle, or LPS to simulate the inflammatory conditions of labour. Vascular endothelial growth factor (VEGF) is a growth factor which is regulated by hypoxia (Dibbens et al. 1999; Popovici et al. 1999) and its expression in the explants was examined to confirm hypoxic conditions.

5.2. METHODS

5.2.1. Recruitment of patients

Six non-labouring women were recruited to donate myometrial biopsies at elective caesarean section and informed, written consent was obtained as described in section 2.2.1. The women were all undergoing elective caesarean section in an uncomplicated, singleton pregnancy, at term.

5.2.2. Sample collection

Myometrial biopsies were taken from the upper margin of the transverse lower uterine segment incision, after delivery of the baby, as described in section 2.1.5. Biopsies were taken by the operating surgeon using either curved mayo scissors or a scalpel and were full thickness, up to 1cm width and 2cm length. The biopsies were placed into RPMI culture medium, transported to the laboratory and used immediately in the explant culture experiment.

5.2.3. Explant culture

The myometrial biopsies were washed three times with PBS and then the decidua was dissected off and the tissue was divided into explants of approximately 1-2mm^3 using scissors. The explants were then placed into fresh RPMI and placed into culture at 37°C overnight in order to starve the explants of any remaining blood that may have been residual after washing. The following morning, explants were placed into wells containing 0.5mls RPMI culture medium supplemented with pen/strep (penicillin 5000units and streptomycin 5000µg/ml). Two explants were used per well.

The explants were then placed into culture in six groups, with the following conditions:

- Room air (21% oxygen (O_2), 5% carbon dioxide (CO_2)), 37°C.
- Hypoxic conditions (6% O_2, 5% CO_2), 37°C.
- Very hypoxic conditions (1% O_2, 5% CO_2), 37°C.
- Room air (21% O_2, 5% CO_2), 37°C, medium treated with LPS 100ng/ml.

- Hypoxic conditions (6% O_2, 5% CO_2), 37°C, medium treated with LPS 100ng/ml.
- Very hypoxic conditions (1% O_2, 5% CO_2), 37°C, medium treated with LPS 100ng/ml.

Hypoxic conditions were generated by using hypoxic chambers. The oxygen conditions represent maternal inspired oxygen concentration (21%), likely oxygen concentrations at the feto-maternal interface in normoxic conditions (6%) and hypoxic conditions at the feto-maternal interface (1%).

The explants were cultured for 24 hours. At the end of culture explants were removed from the culture medium and placed into weighed Eppendorf tubes and onto dry ice to snap freeze the tissue. This was performed within the hypoxic chambers where applicable so that tissue was frozen whilst still at the level of oxygenation they had been exposed to in culture. Tissue samples were then stored at -80°C until further use. Culture medium was placed into Eppendorf tubes and stored at -20°C.

5.2.4. RNA extraction, cDNA manufacture and RT-PCR

Total RNA was extracted from the myometrial explants, RNA concentrations were estimated and cDNA was synthesised, as described in section 2.5.

Expression of mRNA for VEGF, ALOX5 and FPR2/ALX was determined using quantitative RT-PCR according to the methods described in section 2.5.4.

Reaction mixes and primers and probes used for ALOX5 and FPR2/ALX were as described in Chapter 3.

VEGF primers and probes were designed to span intron junctions and had the following sequences: forward primer: 5-TACCTCCACCATGCCAAGT-3, reverse primer 5 -TAGCTGCGCTGATAGACAT-3, and probe 5-ACTTCGTGATGATTCTGCC-3.

5.2.5. ELISA for LXA4

Levels of LXA4 were measured in the explant culture media by ELISA, as described in Section 2.3.

The samples were prepared for use in the ELISA by diluting some samples appropriately according to the following method. During the explant culture, inevitably there is some loss of the culture medium by evaporation. In this experiment three different incubators were used, and although all attempts were made to ensure similar experimental conditions, slightly varying humidities within the incubators meant there was some inconsistency of evaporative loss between the different incubators and on different days. Thus, when preparing the samples, the volume of medium that had been left at the end of culture was measured and the samples standardized by diluting the samples that had been exposed to higher levels of evaporative loss to the volume of the sample with the least evaporative loss.

The final values used for statistical analysis were corrected for wet weight of tissue used in each well (pg/ml/mg of tissue).

5.2.6. Statistical analysis

In this experiment, the effect of two variables on the mRNA expression or protein production of a gene of interest is examined. Conventionally when two separate variables may be having an effect, a two-way ANOVA is used which tests what effect each variable has, and also if there is any interaction between the two variables on the measured effect. For a two-way ANOVA to be used, certain conditions must be met, including normal distribution of the data, and equal variance between the data sets. If the data are not normally distributed, a non-parametric test must be used. In this experiment the numbers of biological replicates was small (n=6) and, as is common when using human tissue samples, variation within groups was wide for some of the data sets. D'Agostino-Pearson tests of normality were used to determine whether parametric or non-parametric testing was more appropriate and as it was demonstrated that the data could not be shown to be normally distributed, non-parametric testing was chosen to analyse effect of the two experimental variables.

A Friedman test with Dunn's multiple comparison post test was used to compare relative mRNA expression of the genes of interest or levels of LXA4 in the culture medium. The Friedman test is used for non-parametric, matched data. As explained in the previous paragraph, these data were assumed to be non-parametric, and a test for matched data was chosen because the same patient's myometrium was exposed to each experimental condition for each of the biological replicates. All calculations were carried out using GraphPad Prism 5.02 (GraphPad software, CA, USA).

5.3. RESULTS

5.3.1. Relative mRNA expression of VEGF in myometrial explants increased with decreasing concentration of oxygen

Relative mRNA expression of VEGF was measured in the cultured myometrial explants after 24 hours of culture. There was a significant increase in VEGF expression as the level of oxygenation decreased, both in the explants that were treated with LPS and the explants which had no treatment (Figure 5.1). These changes were significant when comparing untreated 21% O_2 and 1% O_2 ($p<0.01$, median fold change 4.5, n=6) and when comparing LPS-treated 21% O_2 and 1% O_2 ($p<0.05$, fold change 3.1, n=6).

Treatment with LPS made no significant difference to relative VEGF mRNA expression at any of the three oxygenation conditions.

Upregulation of VEGF is a recognized consequence of culture in hypoxic condition and in this experiment is used to demonstrate that the myometrial explants were being cultured in hypoxic conditions and were responding appropriately to this.

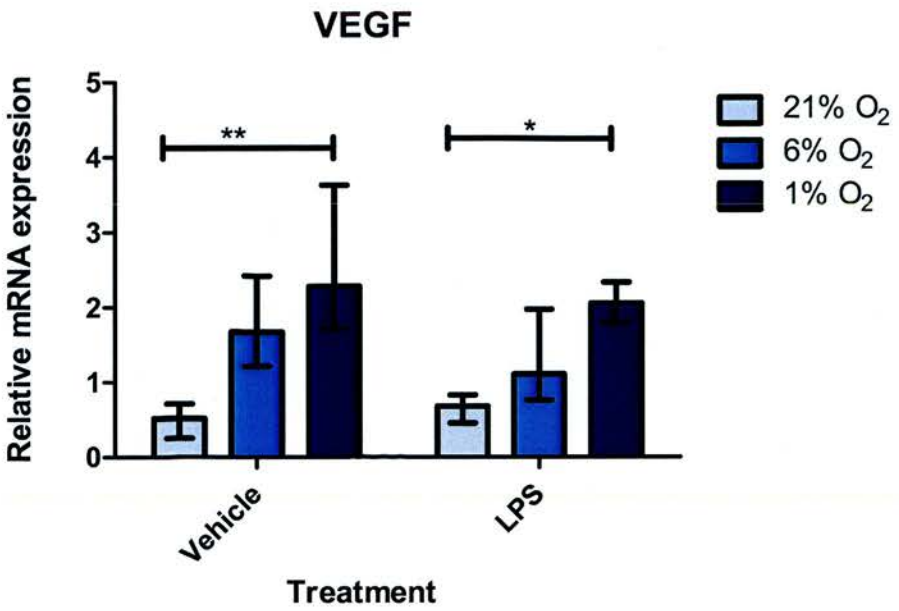


Figure 5.1 VEGF mRNA expression in myometrial explants

Relative mRNA expression of VEGF in myometrial explants cultured for 24 hours in varying oxygen concentrations, with or without LPS treatment. VEGF expression increases with decreasing concentration of oxygen, both when cultured in vehicle (median fold change between 21% and 1%=4.5) or with LPS (median fold change between 21% and 1%=3.1). At any given oxygenation, LPS did not affect expression levels when compared to vehicle, n=6, Friedman test with Dunn's multiple comparison post test, p=0.0002. Data are presented as median with interquartile ranges. *p<0.05, **p<0.01.

5.3.2. Relative mRNA expression of ALOX5 in myometrial explants decreased with decreasing concentration of oxygen when treated with LPS 100ng/ml, but not when treated with vehicle

Relative mRNA expression of ALOX5 mRNA was measured in the myometrial explants following 24 hours of culture (Figure 5.2). The concentration of oxygen had no effect on expression of ALOX5 in myometrial tissue treated with vehicle. However, in the group treated with LPS, expression of ALOX5 was significantly reduced with decreasing oxygen concentration between 21% O_2 and 1% O_2 ($p<0.05$, median fold change 2.7, n=6).

When comparing the effect of LPS with vehicle on relative mRNA expression of ALOX5 at each oxygen concentration, there was a significant difference only at the lowest oxygen concentration, 1% O_2 ($p<0.05$, median fold change 2.5, n=6).

Expression of the other enzymes involved in the synthesis of LXA4, ALOX12 and ALOX15, was not measured because prior work (see Chapter 3) had demonstrated either undetectable or very low expression of these enzymes in myometrial explants.

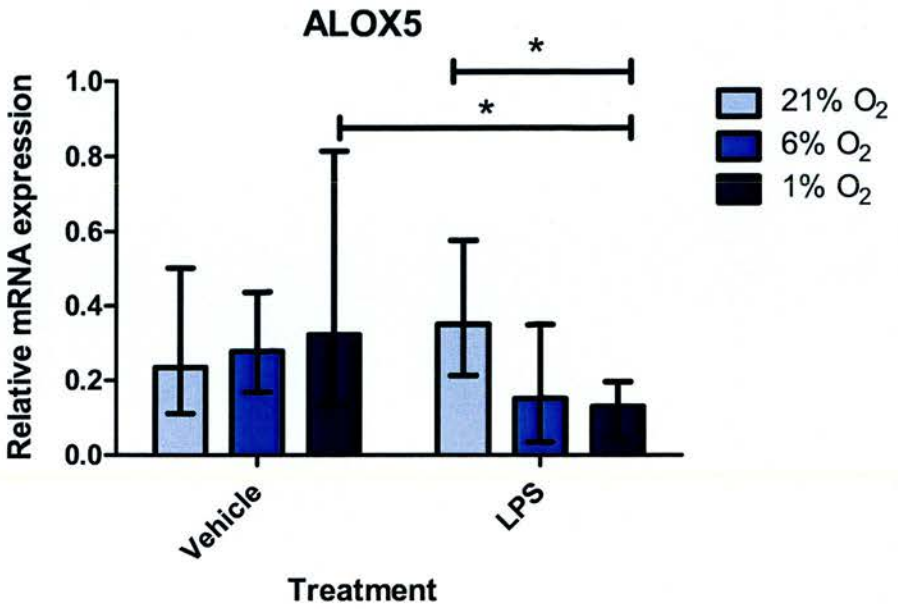


Figure 5.2 ALOX5 mRNA expression in myometrial explants

Relative mRNA expression of ALOX5 in myometrial explants cultured for 24 hours in varying oxygen concentrations, with or without LPS treatment. When treated with vehicle, oxygen concentration does not affect relative ALOX5 mRNA expression. When treated with LPS, ALOX5 expression decreases with decreasing oxygen concentration (median fold change between 21% and 1%=2.7). When comparing the effect of LPS with vehicle on relative mRNA expression of ALOX5 at each oxygen concentration, there was a significant difference only at the lowest oxygen concentration, 1% O_2 ($p<0.05$, median fold change 2.5, n=6). n=6, Friedman test with Dunn's multiple comparison post test, p=0.0002. Data are presented as median with interquartile ranges. *P <0.05.

5.3.3. Relative mRNA expression of FPR2/ALX was not significantly different when treated with LPS or in different oxygen concentrations.

Relative mRNA expression of FPR2/ALX was measured in the cultured myometrial explants. There were no significant differences when comparing the six groups. Thus, expression of FPR2/ALX was not statistically significantly different at different oxygenations in either the group treated with vehicle or the group treated with LPS. The data suggest a trend towards decreasing expression of FPR2/ALX with decreasing oxygenation in the group treated with vehicle but this was not statistically significant.

When comparing the effect of LPS treatment, there was no significant difference of FPR2/ALX expression at any of the oxygen concentrations. (Figure 5.3).

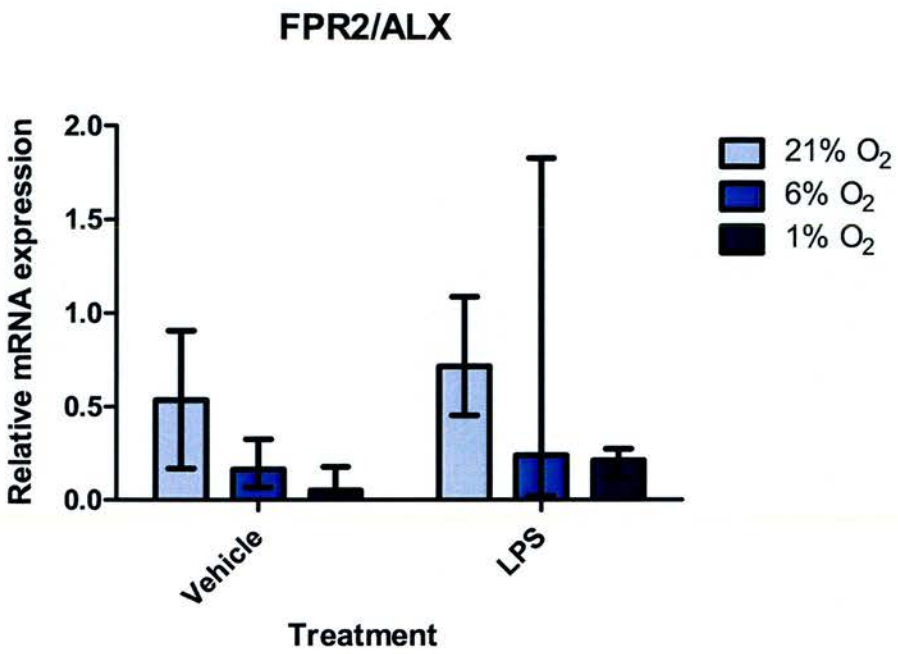


Figure 5.3 FPR2/ALX mRNA expression in myometrial explants

Relative mRNA expression of FPR2/ALX in myometrial explants cultured for 24 hours in varying oxygen concentrations, with or without LPS treatment. There is no statistically significant difference between the six groups. n=6, Friedman test with Dunn's multiple comparison post test. Data are presented as median with interquartile ranges.

5.3.4. There was no significant difference in LXA4 concentration in the culture medium of explants cultured for 24 hours at 21%, 6% or 1% O_2, and with either vehicle or LPS treatment.

ELISA analysis of the culture medium from the six groups of explants demonstrated that LXA4 was present in the medium from these experiments (Figure 5.4). It was not present in culture medium alone that had not had myometrial explant in it and thus the LXA4 must have been produced by the explants. There were six replicates in this experiment i.e. the experiment was repeated on myometrium from six different women. In one of the replicates, the sample reading was out of range (lower end of range) for five out of the six experimental conditions. This sample was the first of the experiments to have been carried out. Later some of the samples used in this experiment were included in another LXA4 ELISA, and on this occasion no LXA4 was detected in the culture medium, despite having been demonstrated in this, previous, experiment. One explanation for this is that LXA4 is degraded quickly and that the second freeze-thaw cycle may have contributed to degradation of the protein so that by the time a second ELISA was performed, none was measurable. This is a potential problem with this experiment as the protein being measured is so fragile. A possible reason for the first replicate's samples being almost all unmeasurable is that these samples were the oldest. This replicate was excluded from analysis and thus the subsequent analysis was performed on a sample size of 5.

There was no significant difference in the concentrations of LXA4 found in the medium between any of the six experimental condition groups.

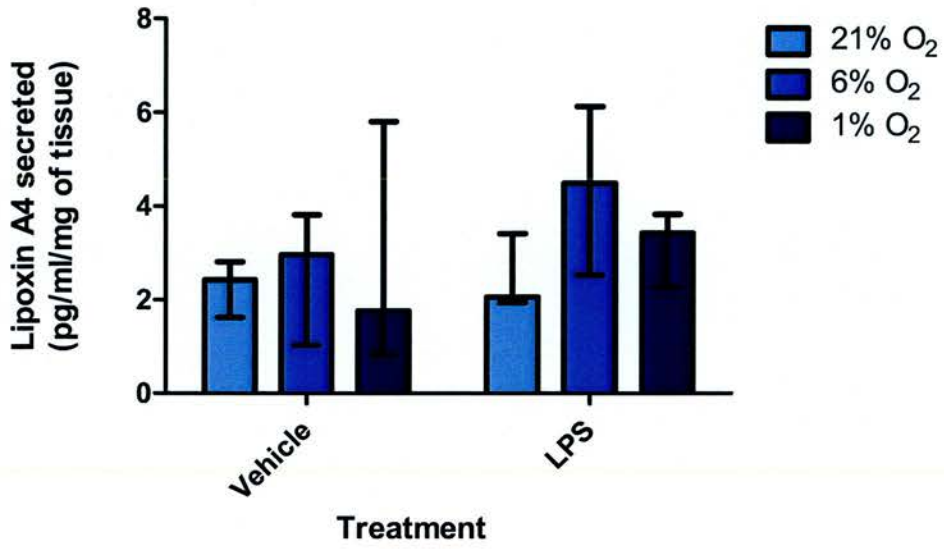


Figure 5.4 LXA4 levels in myometrial explant culture medium

Level of LXA4 detected in the culture medium of myometrial explants cultured for 24 hours in varying oxygen concentrations. There is no statistically significant difference in LXA4 level in any of the six groups. n=5, Friedman test with Dunn's multiple comparison post test. Data are presented as median with interquartile ranges.

5.4. DISCUSSION

The data in this chapter has shown that:

- Relative mRNA expression of VEGF in myometrial explants cultured for 24 hours increased with decreasing concentration of oxygen, both when treated with LPS 100ng/ml, and when treated with vehicle alone.

- Relative mRNA expression of ALOX5 in myometrial explants cultured for 24 hours decreased with decreasing concentration of oxygen when treated with LPS 100ng/ml.

- When treated with vehicle alone, relative mRNA expression of ALOX5 in myometrial explants cultured for 24 hours was not affected by decreasing concentrations of oxygen.

- Neither oxygen tension nor LPS treatment had any effect on myometrial LXA4 release into culture medium.

- Relative mRNA expression of FPR2/ALX in myometrial explants cultured for 24 hours was not statistically significantly affected by either oxygen tension or LPS treatment. There did appear to be a trend towards reduced expression with reducing oxygen concentrations, which would benefit from further experimentation with greater numbers.

In this work I have studied for the first time what effect hypoxia might have on an anti-inflammatory and pro-resolution mediator, LXA4, in human pregnant myometrium. The samples used for this experiment were taken from non-labouring patients and so co-treatment with LPS was designed to upregulate inflammatory mediators and to allow the effect of any anti-inflammatory / pro-resolution mediators to become apparent.

Hypoxic treatment conditions caused an upregulation of mRNA expression of VEGF. This was an expected finding and was used in this experimental model to demonstrate an appropriate, dose-related response to hypoxia by the myometrial explants, as has been illustrated in other tissues (Dibbens et al. 1999; Popovici et al. 1999).

Although it is well recognised that the expression of VEGF is regulated by hypoxia in other systems including endometrium (Sharkey et al. 2000), this is the first time that it has been specifically reported that VEGF could be inducible by hypoxic culture conditions in myometrial tissue. In HUVEC cells, LXA4 regulates the effects of VEGF via an effect on the receptor VEGFR2 and inhibits VEGF-stimulated expression of IL-6 and TNF-α, whilst augmenting VEGF-stimulated production of IL-10 (Baker et al. 2009). This study has not attempted to demonstrate functional effects of LXA4 on VEGF expression.

As one of the enzymes involved in the synthesis of LXA4 it might be anticipated that increased expression of ALOX5 might result in increased production of LXA4. The data in this chapter demonstrate that ALOX5 mRNA expression decreases with decreasing concentrations of O_2, but only when treated with LPS. When treated with vehicle there was no difference in expression of ALOX5 at any of the three O_2 concentrations used. Culture with LPS has been demonstrated to reduce the expression of ALOX5 in astrocytes (Johann et al. 2008) and down-regulate ALOX5 activity (Brock 2002). In my data, ALOX5 mRNA expression was downregulated by LPS at 1% O_2, but a significant effect was not shown at the other levels of oxygenation. This suggests that the braking effects of LXA4 on inflammation are likely to be attenuated in hypoxic conditions, i.e. that hypoxia amplifies inflammation in the myometrium.

ALOX 12 and ALOX 15 expression were not measured in this experiment. This was because previous work had demonstrated very low or undetectable levels of mRNA expression of these genes in similar myometrial explants. The cell types present in this explant experiment are not identical to those in vivo, and specifically, it is possible that neutrophil populations are different, particularly as it is known that

labouring myometrium contains an influx of invading neutrophils (Osman et al. 2003). It is possible that this may have influenced the data produced by this experiment.

Myometrial explants in each of the experimental conditions examined in this study produced LXA4 that was present at detectable levels in the culture medium, but there was no significant difference in LXA4 levels when comparing any of the experimental conditions. It has been observed previously by our laboratory that myometrial explants in culture secrete LXA4 into the culture medium (Maldonado-Perez et al. 2010). In the data presented in this chapter it appears as though levels of secreted LXA4 were higher than was reported in the previous study, by an order of magnitude of around 2 to 3. The two experiments were performed in very similar ways, so the reason for this is unclear. However, in the study described in this chapter it was noted in subsequent experiments using the same media that LXA4 levels declined significantly after just one further freeze-thaw cycle (data not shown) and therefore it is possible that the observed discrepancies between the two studies are due to differences in handling of the culture medium during the ELISA experiment itself. All media in the experiments described here were handled identically in terms of being frozen and stored immediately after culture and then being used in the ELISA immediately after defrosting and so it is reasonable to assume any degradation of the protein that may have occurred was consistent throughout the samples. However, it is not known at what rate degradation of LXA4 was occurring in the samples during the 24 hours of culture and whether this was affected by hypoxia. Equally, it is not known whether substrate for LXA4 synthesis could have been exhausted in the experiment, whether this may have varied depending on the experimental conditions and at what time in the experiment this may have occurred. Given that ALOX5 is required for LXA4 synthesis, it would be expected that the effect of ALOX5 inhibition would be to reduce LXA4 production and that less LXA4 would be detected in the culture media of explants cultured in hypoxia conditions. It is uncertain whether the discrepancies between LXA4 and ALOX5 expression are due to LXA4 degradation, or whether they reflect continued LXA4 production despite a decrease in ALOX5. Time course experiments exploring

the degradation of a known level of LXA4 in the presence of absence of hypoxia may have helped to answer this question.

The time frame chosen for this experiment was 24 hours. This was chosen based on previous work produced in our laboratory which had demonstrated that LXA4 was produced from myometrial explants at both 4 and 24 hours and that there was no difference in the levels produced at these timepoints. The longer timepoint was chosen to allow time for any genomic changes to have taken place and be observable via the quantitative RT-PCR data. Ideally, a number of timepoints would have been chosen to produce a greater picture of any changes, and their immediacy, but only a limited number of explants can be obtained from each patient sample.

The effect of hypoxia on FPR2/ALX expression or function has not been previously tested. In this experiment, mRNA expression of FPR2/ALX was not significantly affected by either culture with LPS, or by varying levels of oxygen concentration. The data do appear to show a trend towards downregulation of FPR2/ALX with reduced oxygen concentration in the group treated with vehicle, although this was not statistically significant. This may be due to a combination of a small sample size and data with high standard errors, leading to a type II error. A larger sample size would help to answer this question more reliably. If there were a change in FPR2/ALX expression with reduced oxygenation, this could represent a mechanism by which pro-resolution and anti-inflammatory mediators may have altered effects in hypoxic conditions.

These data demonstrate that whilst we found no change in production of LXA4 in myometrium in vitro in hypoxic conditions, there is a decrease in one of its synthetic enzymes ALOX5. If a reduction in ALOX5 leads to decreased LXA4 over a longer period of time, then there is a potential mechanism by its function may be altered in myometrium in hypoxic conditions. It has not been demonstrated what functional effect this may be, or what effect might subsequently be observed in vivo in parturition, although it might lead to an attenuation in the inhibitory effect of LXA4 on IL-6 and IL-8 production in myometrium in vitro (Maldonado-Perez et al. 2010) contributing to an overall pro-inflammatory environment.

Future work could include examining the production of pro-inflammatory mediators such as IL-6, IL-8 and COX-2 by the myometrial explants. A study of myometrial smooth muscle cells by Helmer et al showed no difference in production of IL-6 and IL-8 when cultured in hypoxia (Helmer et al. 2002), although the same study reported no difference in IL-6 and IL-8 when stimulated with LPS. This is contrary to our own laboratory's findings on stimulating myometrial tissue explants with LPS (Maldonado-Perez et al. 2010) and therefore different results may well be observable on stimulating with hypoxia.

Given that myometrial contractility is affected by hypoxia, it would be interesting to observe what, if any, effect culture with LXA4 has on myometrial contractility and if this were altered by culturing in different oxygen conditions. Such data may be helpful in developing strategies to treat ineffective labour and reduce the burden of caesarean section on mothers and the health service.

6. The role of Annexin A1 in the human pregnant reproductive tissues

6.1. INTRODUCTION

Annexin A1 (ANXA1) is a protein with powerful anti-inflammatory and pro-resolution properties. ANXA1 is found throughout the body in a variety of cell types. In the blood, neutrophils and monocytes are the predominant cellular source of ANXA1 (Perretti et al. 1996). It is also expressed by epithelial cells in the lung, gut and kidney, and by fibroblasts (Errasfa et al. 1985; Vervoordeldonk et al. 1994; Solito et al. 1998; Babbin et al. 2006). In neutrophils, ANXA1 is found within the cell cytoplasm, from where it is mobilised when the cell is activated, and secreted from the cell surface (Perretti et al. 2000). ANXA1 subsequently can function in an autocrine or paracrine fashion to activate signalling of its receptor, FPR2/ALX (Walther et al. 2000). Related to ANXA1 are Ac2-26 and Ac9-25, short peptides corresponding to the N-terminal sequence of ANXA1, which have similar functions and signal not only through FPR2/ALX, but also through FPR1, a closely related GPCR from the same family of receptors (Hayhoe et al. 2006). The anti-inflammatory and pro-resolution actions of ANXA1 include reduction of transmigration of neutrophils (Perretti et al. 1996; Walther et al. 2000), increase in the detachment of neutrophils adherent to endothelial cell layers (Lim et al. 1998), an increase in neutrophil apoptosis (Solito et al. 2003), and phagocytosis of apoptotic neutrophils (Scannell et al. 2007).

Glucocorticoids have been identified as an important regulator of ANXA1 and appear to increase its expression by both genomic and non-genomic pathways. Glucocorticoid administration has been demonstrated to cause an increase in leucocyte cell surface expression of ANXA1 within 30 minutes in humans (Goulding et al. 1990). With regard to upregulation of transcription, a specific glucocorticoid-response element has not been found on ANXA1 and it is possible that glucocorticoids indirectly bring about upregulation, perhaps via IL-6 (Solito et al. 1998). Furthermore, glucocorticoids have also been shown, in human monocytes, to induce expression of the receptor, FPR2/ALX, both at an mRNA level and at the protein level (Sawmynaden and Perretti 2006).

Glucocorticoids play a crucial role throughout human pregnancy, with effects on implantation (Arcuri et al. 1997), placental development (Malassine and Cronier 2002), fetal brain development, fetal lung, liver and gastrointestinal maturation (Challis et al. 2001) and ultimately, in parturition itself. Cortisol increases prostaglandin synthesis and actions, which has pro-labour effects including inducing cervical ripening, myometrial contractility and facilitating membrane rupture and indirect effects on the inflammatory cascades encountered during human parturition. However, excess cortisol levels can have a deleterious effect in pregnancy, observed in restriction of fetal growth (Seckl and Meaney 2004), and also longer term fetal programming effects (Seckl 2004).

11β-hydroxysteroid dehydrogenase (11βHSD) is an enzyme existing in two isoforms, 11βHSD1 and 11βHSD2 (Brown et al. 1993), which interact in a so-called cortisol-cortisone shuttle (Edwards and Stewart 1991). 11βHSD2 acts as an oxidase, converting the active hormone cortisol, to its inactive form cortisone. 11βHSD1 on the other hand has some oxidative function, but mainly functions as a reductase and converts inactive cortisone to active cortisol.

Maternal plasma cortisol levels in pregnancy are in the order of ten times greater than fetal cortisol levels (Shams et al. 1998), a state which is maintained by the actions of 11βHSD2 in the placenta (Murphy et al. 1974; Blasco et al. 1986; Stewart et al. 1995), acting to convert the active glucocorticoid, cortisol, to the inactive form, cortisone. Thus 11βHSD2 has a feto-protective role within the placental unit (Blasco et al. 1986; Stewart et al. 1995). However, 11βHSD1 is also produced in the human placental unit, more notably in the decidua and fetal membranes (Sun et al. 1997), which is likely to increase local production of cortisol from cortisone. Cortisol levels increase during human pregnancy towards term in the amniotic fluid (Murphy et al. 1975), and this is presumed to be increased production from the fetal adrenal. Indeed, in sheep, this increased cortisol production by an increasingly mature fetus is likely the main initiator of parturition, although the human scenario is much more complex (Golightly et al. 2010). Increased local production of cortisol towards term or in labour may contribute further to the initiation of parturition, both normal, and preterm via increased production of prostaglandins, as described above. However,

increased cortisol production may also have an effect on the anti-inflammatory and pro-resolution mediator ANXA1 in late gestation and parturition, given the influence of glucorticoids on ANXA1 (Perretti and D'Acquisto 2009).

It has been observed that the mRNA expression of ANXA1 is upregulated in pregnant myometrium when compared to non-pregnant myometrium (Rehman et al. 2003). Expression has also been reported in placenta (Bennett et al. 1994) and fetal membranes (Lynch-Salamon et al. 1992; Bennett et al. 1994). However, relatively little work is published on its potential role within pregnancy or the puerperium. ANXA1 null mice conceive, gestate their litters, labour normally and deliver apparently normal pups at an appropriate time (Hannon et al. 2003). This observation is in contrast to the profoundly abnormal response to other inflammatory events that such null mice produce (Roviezzo et al. 2002). There are differences in the annexin systems between mice and humans, therefore it is not entirely reliable to extrapolate these observations to the human events. Given that human parturition is an inflammatory event, it may be that ANXA1 has a role in this process, perhaps by contributing to the delicate balance between the continuation of pregnancy and the ensuing inflammation of labour.

Aims

The research in this chapter aims:

- to localise the ANXA1 protein within myometrium and placenta, and compare that localisation between labouring and non-labouring tissues
- to explore the relative mRNA expression of ANXA1 and FPR1 in labouring and non-labouring tissues of the feto-placental unit
- to explore to what extent expression of the 11βHSD enzymes is altered in term labouring and non-labouring tissues
- and to study what effect altered cortisol levels may have on the ANXA1-FPR1/2 system.

6.2. METHODS

6.2.1. Recruitment of patients

6.2.1.1. Samples for use in comparison of labouring vs non-labouring tissue by quantitative RT-PCR:

Groups of labouring (n=11) and non-labouring women (n=11) were recruited and informed, written consent was obtained to provide tissue samples as described in section 2.2.1. Inclusion and exclusion criteria for these groups are described in section 2.2.2.1.

6.2.1.2. Samples for use in explant tissue culture:

Five non-labouring women were recruited to donate myometrial biopsies at elective caesarean section and informed, written consent was obtained as described in section 2.2.1. The women were all undergoing elective caesarean section in an uncomplicated, singleton pregnancy, at term.

6.2.2. Sample collection

6.2.2.1. Samples for use in comparison of labouring vs non-labouring tissue by quantitative RT-PCR:

Samples of myometrium, placenta, amnion and chorio-decidua were taken at the time of elective or emergency caesarean section, as described in section 2.1.5.1.

6.2.2.2. Samples for use in explant tissue culture:

Myometrial biopsies were taken from the upper margin of the transverse lower uterine segment incision, after delivery of the baby, as described in section 2.1.5.1. Biopsies were taken by the operating surgeon using either curved mayo scissors or a scalpel and were full thickness, up to 1cm width and 2cm length. The biopsies were placed into RPMI culture medium, transported to the laboratory and used immediately in the explant culture experiment.

6.2.3. Immunohistochemistry

ANXA1 was localised in labouring and non-labouring myometrium, placenta and fetal membranes by immunohistochemistry.

The immunohistochemistry protocol used for determining the localisation of ANXA1 is described in section 2.4 and was performed on sections cut from myometrial, placental and membrane biopsies, taken either at elective or emergency caesarean section, as described in section 2.1.5.1.

6.2.4. Myometrial explant culture with cortisol

The myometrial biopsies were washed three times with PBS and then the decidua was dissected off and the tissue was divided into explants of approximately 1-2mm^3 using scissors. The explants were then placed into fresh RPMI and placed into culture at 37°C overnight in order to starve the explants of any remaining blood that may have been residual after washing. The following morning, explants were placed into RPMI culture medium supplemented with P/S (penicillin 5000units and streptomycin 5000µg/ml) and containing one of the following treatments:

1. Vehicle.

The cortisol used was suspended in ethanol, therefore ethanol was added to the culture medium at the same concentration as the highest dose of cortisol used. Stock solution of cortisol was 10^{-3}M, therefore to make a solution of 10^{-6}M, a dilution of 1µl in 1000µl RPMI+P/S was used. Hence for the vehicle, 1µl of cortisol was added per 1000µl of culture medium.

2. Cortisol 10^{-6}M

As described above, stock solution of cortisol was 10-3M so a dilution of 1µl per 1000µl RPMI+P/S was used to achieve a concentration of 10-6M.

3. Cortisol 10^{-7}M

4. Cortisol 10^{-8}M

5. Cortisol 10^{-9}M

For the subsequent doses in treatments 3, 4 and 5, treatment 2 was sequentially diluted with further RPMI+P/S.

In each experiment, the explants and culture medium were placed into wells on a 24 well plate. In each well, 0.5mls of culture medium were used and 2 explants were placed. For each treatment 3 triplicate wells were used.

The plate was placed into culture in an incubator at 37°C for either 6 or 24 hours. After this time, the plate was removed from culture. Myometrial explants were removed from culture and placed into a pre-weighed 2ml Eppendorf tube and immediately placed onto dry ice to snap freeze the sample. Samples were then transferred for storage at -80°C until further analysis. Forceps were washed between each separate well.

6.2.5. Quantitative RT-PCR

Quantitative RT-PCR was used to determine relative mRNA expression of ANXA1, FPR1, 11βHSD1 and 11βHSD2 in labouring and non-labouring tissues.

Total RNA was extracted from the biopsies of myometrium, placenta, amnion and chorio-decidua, RNA concentrations were estimated and cDNA was synthesised, as described in section 2.5. Quantitative RT-PCR was performed as described in section 2.5. Specific reaction mix composition and primers and probes used are detailed below.

6.2.5.1. Annexin A1

Expression of ANXA1 mRNA was determined using a TaqMan ® Gene Expression Assay (Hs00167549_m1, Applied Biosystems) which contains predesigned gene-specific primers and probes.

6.2.5.2. FPR1

Expression of FPR1 mRNA was determined was determined using TaqMan ® Gene Expression Assay system (Hs00181830_m1, Applied Biosystems) which contains predesigned gene-specific primers and probes.

6.2.5.3. 11βHSD1

Expression of 11βHSD1 mRNA was determined was determined using TaqMan ® Gene Expression Assay system (Hs00181830_m1, Applied Biosystems) which contains predesigned gene-specific primers and probes.

6.2.5.4. 11βHSD2

Expression of 11βHSD2 mRNA was determined was determined using TaqMan ® Gene Expression Assay system (Hs00181830_m1, Applied Biosystems) which contains predesigned gene-specific primers and probes.

The reaction mix used gave a final volume of 15µl per well, containing 7.5 µl 2x Express Mastermix, 0.75µl primer/probe gene expression assay mix, 0.225µl 18S primer/probe assay mix, 0.375µl cDNA and 6.15µl RNase-free, DNase-free sterile water.

6.2.5.5. Quantitative RT-PCR of ANXA1, FPR1 and FPR2/ALX in explant samples

Quantitative RT-PCR was used to determine relative mRNA expression of ANXA1, FPR1 and FPR2/ALX in the myometrial samples from the explant experiment described in 6.2.4.

Total RNA was extracted from the myometrial explants, RNA concentrations were estimated and cDNA was synthesised, as described in section 2.5. Quantitative RT-PCR was performed as described in section 2.5. Specific reaction mix composition and primers and probes used are given for ANXA1 and FPR1 in section 6.2.4 and for FPR2/ALX below.

FPR2/ALX

Expression of FPR2/ALX mRNA was determined using primers designed by Dr David Maldonado-Perez using the ProbeFinder version 2.42 for Human (Roche Applied Science, Burgess Hill, UK). The sequences were: forward 5'-GCACACAGGAAAAGGAGCTTA, reverse 5'-AGCCAGCAGACTCATAGGACA. The corresponding probe from the Universal

Probe Library Set was used with each pair of primers. PCR was performed with a final volume of 25 µl per well, containing 12.5µl 2x Mastermix, 0.3µl of each of the forward and reverse primers, 1µl probe, 1.25µl 18S primer/probe assay mix, 1µl cDNA and 8.65µl RNase-free, DNase-free sterile water.

6.2.6. Statistical analysis

A Mann-Whitney test was performed to compare relative gene expression in samples of labouring and non-labouring myometrium, placenta, amnion and chorion. To compare the relative levels of mRNA expression between the four different types of tissue in the labouring group a Friedman test with Dunns Multiple Comparison test was performed. The Friedman test assumes non-parametric data, and that values in each row represent matched data. This test was chosen in view of the fact that the four different types of tissue (i.e. myometrium, placenta, amnion and choriodecidua) were taken from the same eleven women in the labouring group and could therefore be considered as matched samples. The same tests were performed for the data from the non-labouring group. In the explant experiment, paired t-tests were used to compare vehicle and cortisol treatment where only one treatment dose was measures, and where a range of treatment doses were measured, a Friedman test with Dunns Multiple Comparison test was performed. All statistical calculations were carried out using GraphPad Prism 5.02 (GraphPad software, CA, USA).

6.3. RESULTS

6.3.1. Immunohistochemistry of Annexin A1 in labouring and non-labouring myometrium, placenta and fetal membranes

Immunohistochemistry of non-labouring myometrium (Fig 6.1A) demonstrated localisation to myocytes with staining diffusely through the cell. There was also staining evident in the endothelial cells of the blood vessels within the myometrium. Strong staining was seen in neutrophils, which were generally located within the intravascular space. Labouring myometrium (Fig 6.1B) demonstrated a similar staining pattern in that there was staining of the myocytes, endothelial cells and neutrophils.

In non-labouring (Fig 6.2A) and labouring (Fig 6.2B) placenta, there was staining of the syncytiotrophoblasts, with the strongest staining in the brush border of these cells, contacting the maternal circulation. Staining was also seen in endothelial cells of the placental blood vessels, and in neutrophils of the maternal circulation.

In fetal membranes, amnion and chorio-decidua both showed positive staining. Staining was similar in non-labouring (Fig 6.3A) and labouring (Fig 6.3B) samples. ANXA1 localised to the amnionic epithelial cell layer, to the chorionic mesoderm and to trophoblastic cells.

Localisation of FPR2/ALX by immunohistochemistry is described in Chapter 3. It would also have been interesting to perform localisation for FPR1, but efforts to optimise an antibody were not complete in time to perform the experiments for inclusion in this work.

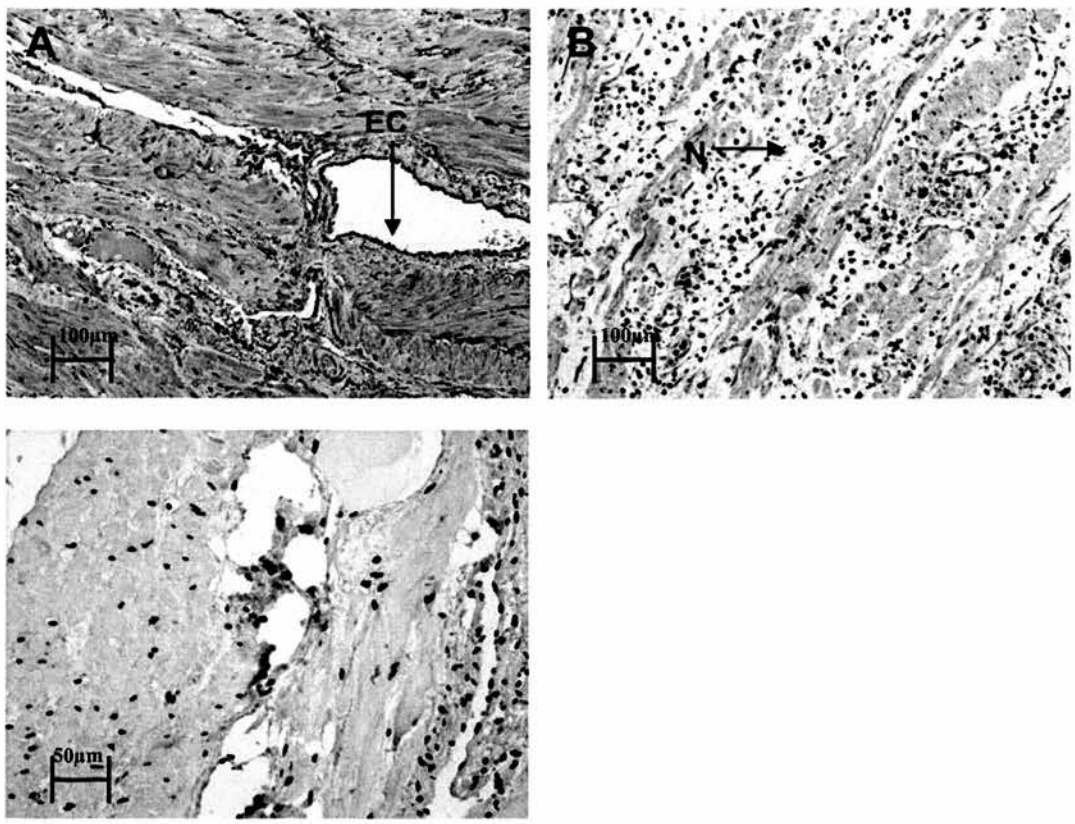


Figure 6.1

Immunolocalisation of ANXA1 in myometrium

Immunohistochemistry demonstrating staining for ANXA1 in (A) non-labouring myometrium and (B) labouring myometrium. Negative control is shown below. Staining is seen in myocytes, endothelial cells (EC) and neutrophils (N).

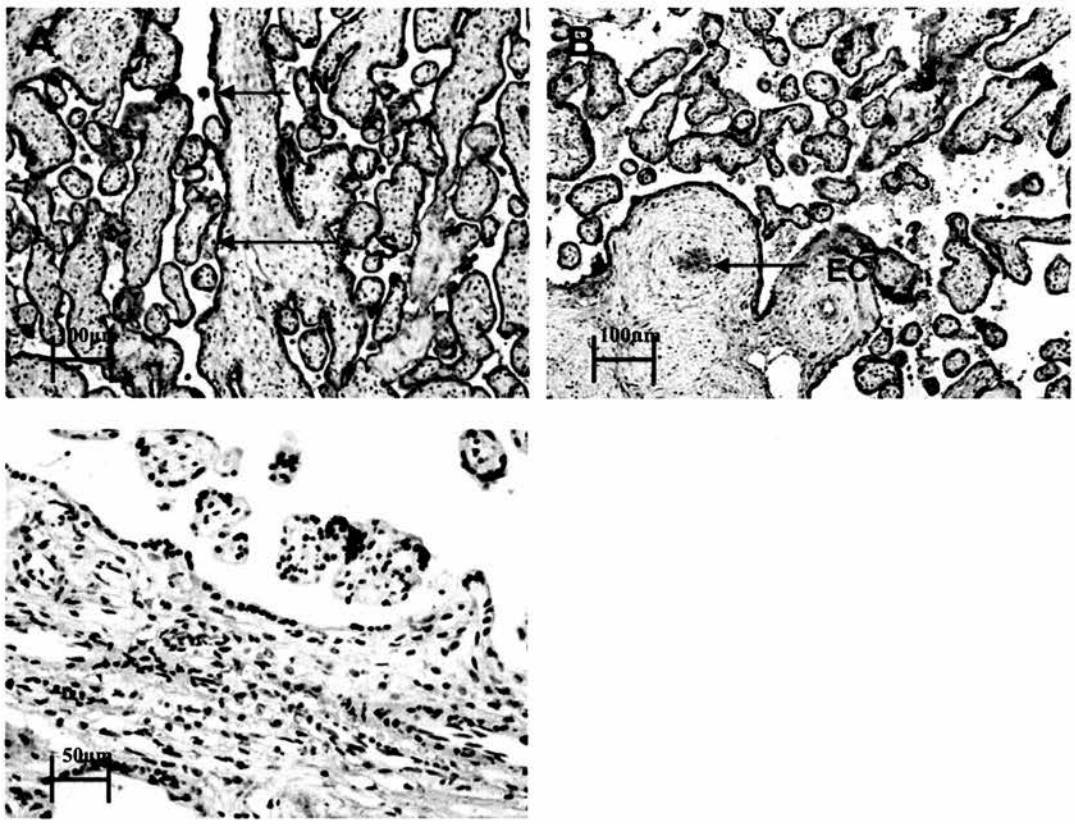


Figure 6.2

Immunolocalisation of ANXA1 in placenta

Immunohistochemistry demonstrating staining for ANXA1 in (A) non-labouring placenta and (B) labouring placenta. Negative control is shown below. Staining is seen in the syncytiotrophoblasts (S), endothelial cells (EC) and neutrophils (N).

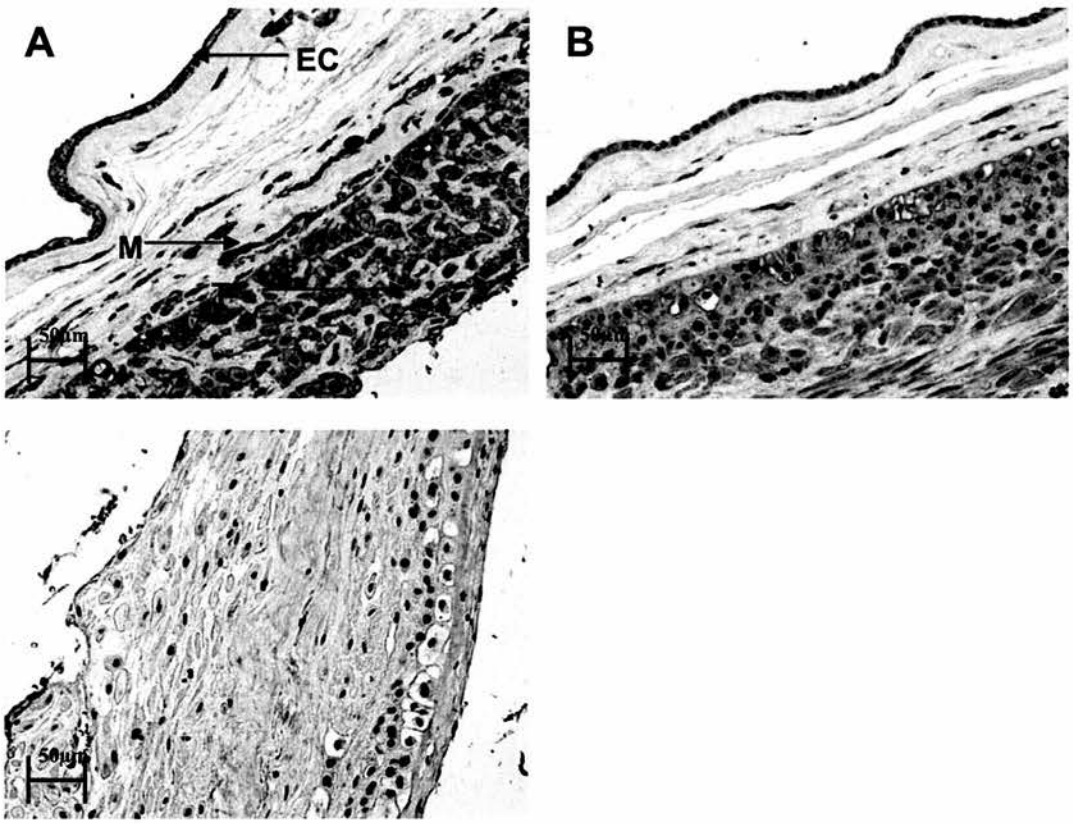


Figure 6.3

Immunolocalisation of ANXA1 in fetal membranes

Immunohistochemistry demonstrating staining for ANXA1 in (A) non-labouring fetal membranes and (B) labouring fetal membranes. Negative control is shown below. Staining is seen in the amnion epithelial cell (EC), in the chorionic mesoderm (M) and in the trophoblastic cells (T).

6.3.2. Relative mRNA expression of Annexin A1 did not differ in labouring and non-labouring myometrium, placenta, amnion and chorio-decidua

When expression was compared between labouring and non-labouring tissue of the same type, there were no significant differences in relative mRNA expression of ANXA1 in labouring and non-labouring myometrium (Figure 6.4, p=0.12); placenta (Figure 6.5, p=0.29); amnion (Figure 6.6, p=0.10) and chorio-decidua (Figure 6.7, p=0.29).

When expression was compared in the non-labouring tissues, relative mRNA expression of ANXA1 was greater in placenta (median fold change of 4.2) and amnion (median fold change of 3.5) than in myometrium ($p<0.0001$, Fig 6.8).

When expression was compared in labouring tissue, relative mRNA expression of ANXA1 was greater in placenta and amnion than it was in myometrium (median fold changes 4 and 4.3 respectively) and in chorio-decidua (median fold changes 2.1 and 2.2 respectively) ($p<0.0001$, Figure 6.9).

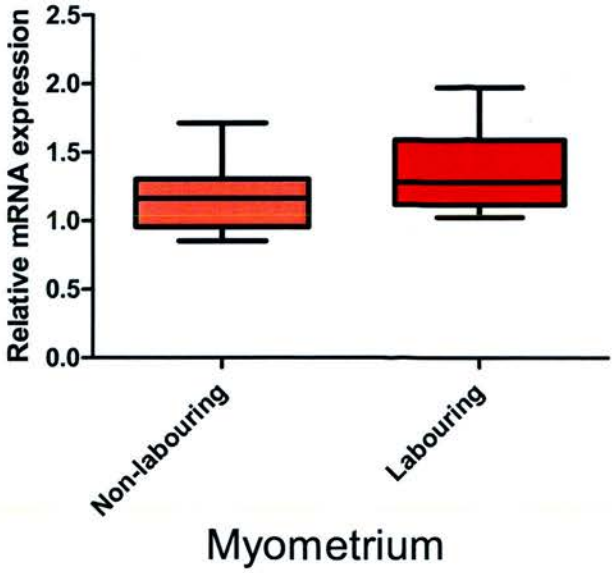


Figure 6.4 ANXA1 mRNA expression in myometrium

Relative mRNA expression of ANXA1 is not significantly different in labouring compared to non-labouring myometrium. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.12, Mann-Whitney test.

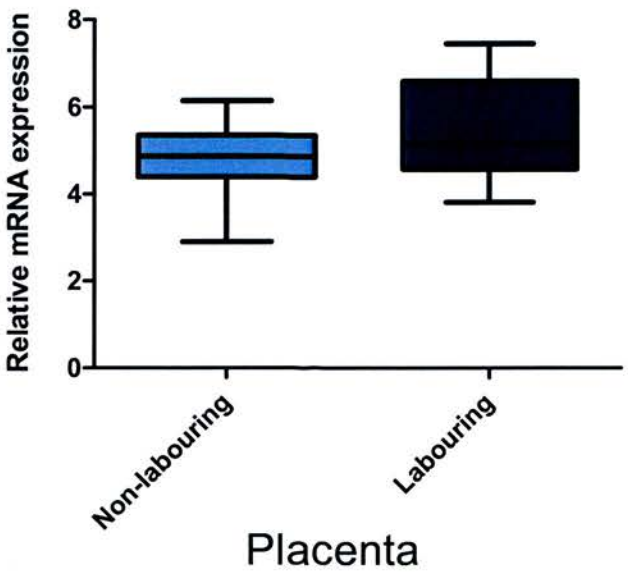


Figure 6.5 ANXA1 mRNA expression in placenta

Relative mRNA expression of ANXA1 is not significantly different in labouring compared to non-labouring placenta. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.29, Mann-Whitney test.

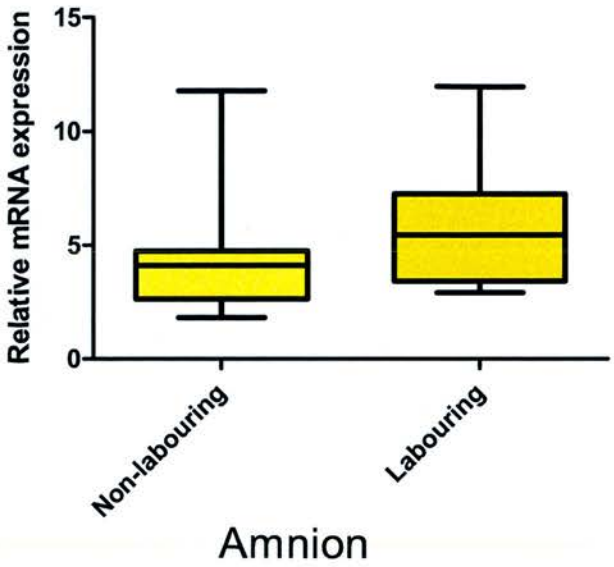


Figure 6.6 ANXA1 mRNA expression in amnion

Relative mRNA expression of ANXA1 is not significantly different in labouring compared to non-labouring amnion. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.10, Mann-Whitney test.

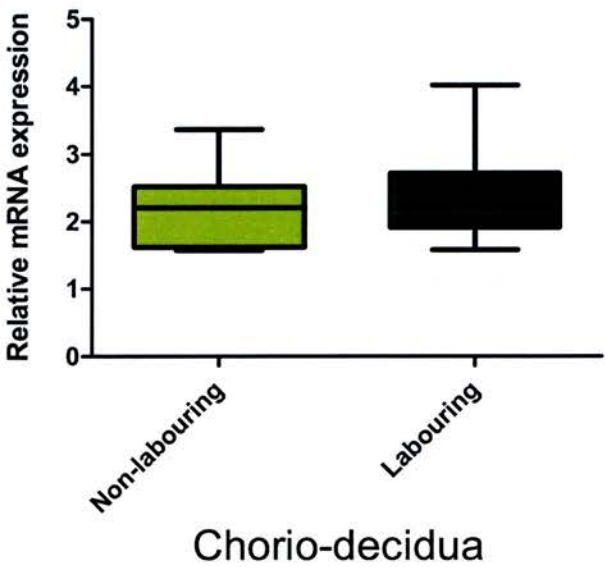


Figure 6.7 ANXA1 mRNA expression in chorio-decidua

Relative mRNA expression of ANXA1 is not significantly different in labouring compared to non-labouring chorio-decidua. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.29, Mann-Whitney test.

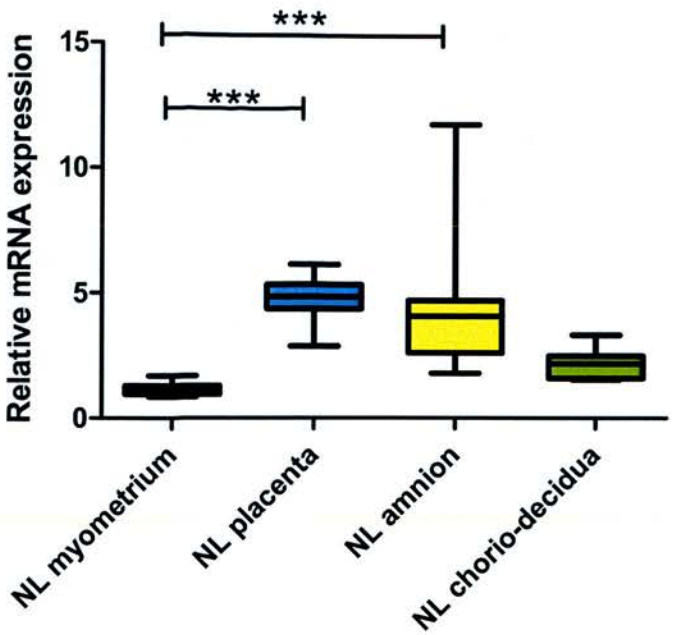


Figure 6.8 ANXA1 mRNA expression in non-labouring tissues

Relative mRNA expression of ANXA1 is greater in non-labouring (NL) placenta and amnion than in myometrium. Data are presented as medians and inter-quartile ranges with min and max whiskers. Friedman test ($p<0.0001$) with Dunns post-test comparison. ***$p<0.001$.

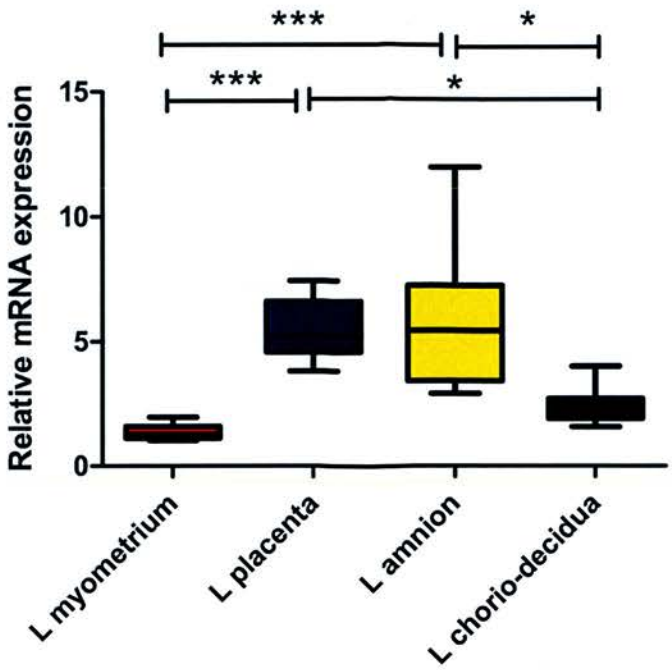


Figure 6.9 ANXA1 mRNA expression in labouring tissues

Relative mRNA expression of ANXA1 is greater in labouring (L) placenta and amnion than in myometrium and chorio-decidua. Data are presented as medians and inter-quartile ranges with min and max whiskers. Friedman test ($p<0.0001$) with Dunns post-test comparison. ***$p<0.001$, *$p<0.05$

6.3.3. Relative mRNA expression of FPR1 was greater in labouring than in non-labouring myometrium, placenta, amnion and chorio-decidua

Relative mRNA expression of FPR1 was greater in labouring myometrium ($p=0.0002$, Fig 6.10), placenta ($p=0.007$, Fig 6.11), amnion ($p=0.018$, Fig 6.12) and chorio-decidua ($p=0.0005$, Fig 6.13) when compared with non-labouring tissue of the same type. Fold changes of the median values were 10.9, 3, 4.3 and 26.2 respectively. Thus the most pronounced fold changes were seen in myometrium and chorio-decidua, as was the case for FPR2/ALX, as reported in Chapter 3.

In both non-labouring (Fig 6.14) and labouring tissues (Fig 6.15) there were no significant differences in relative mRNA expression of FPR1 between the four studied tissues.

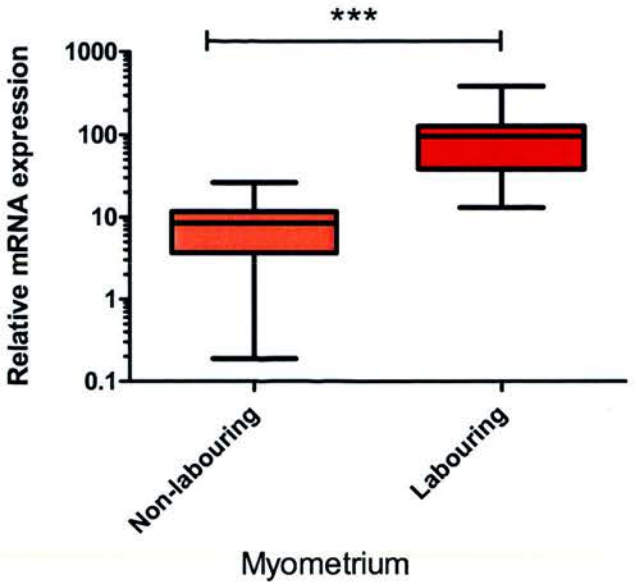


Figure 6.10 FPR1 mRNA expression in myometrium

Relative mRNA expression of FPR1 is significantly greater in labouring compared to non-labouring myometrium (median fold change 10.9). Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.0002, Mann-Whitney test.

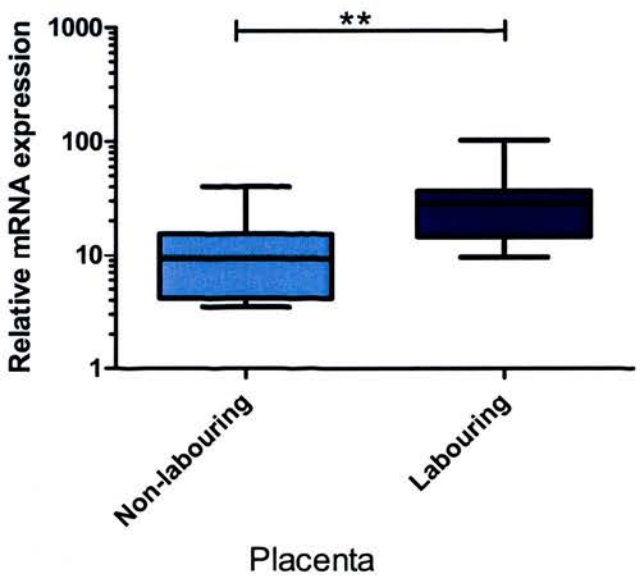


Figure 6.11 FPR1 mRNA expression in placenta

Relative mRNA expression of FPR1 is significantly greater in labouring compared to non-labouring placenta (median fold change 3). Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.007, Mann-Whitney test.

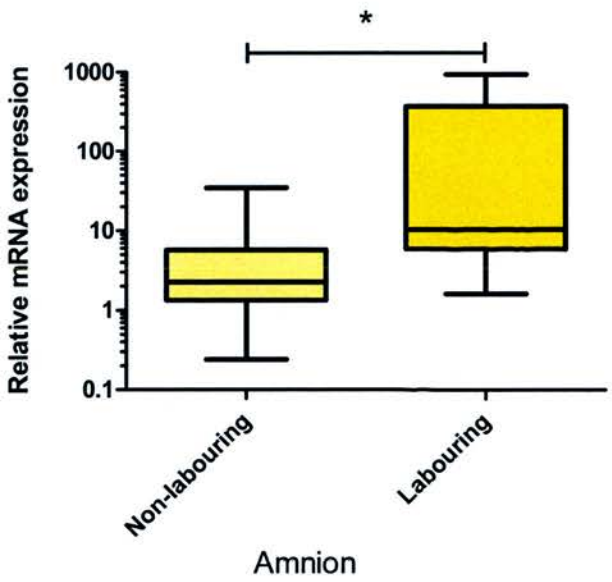


Figure 6.12 FPR1 mRNA expression in amnion

Relative mRNA expression of FPR1 is significantly greater in labouring compared to non-labouring amnion (median fold change 4.3). Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.018, Mann-Whitney test.

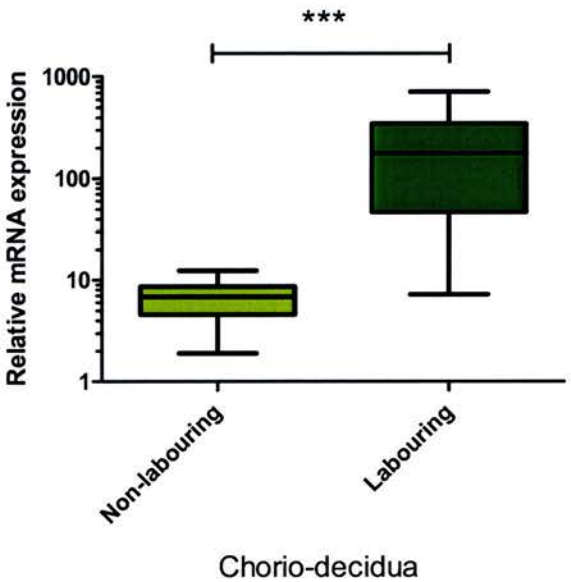


Figure 6.13 FPR1 mRNA expression in chorio-decidua

Relative mRNA expression of FPR1 is significantly greater in labouring compared to non-labouring chorio-decidua (median fold change 26.2). Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.0005, Mann-Whitney test.

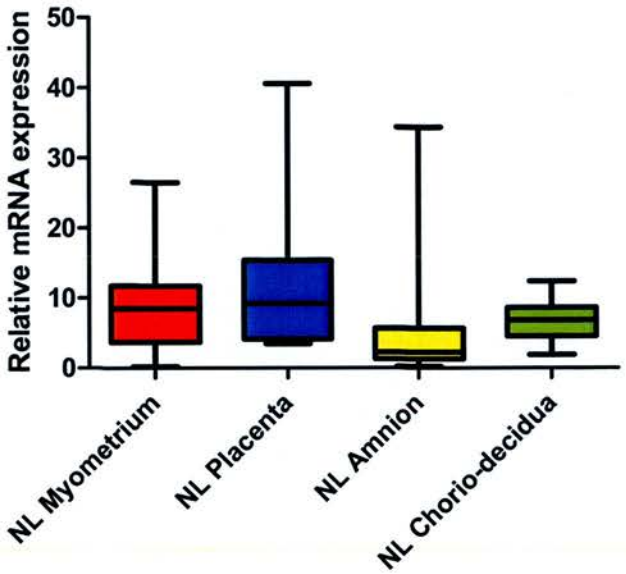


Figure 6.14 FPR1 mRNA expression in non-labouring tissues

Relative mRNA expression of FPR1 was not significantly different in the four non-labouring (NL) tissues of myometrium, placenta, amnion and chorio-decidua. Data are presented as medians and inter-quartile ranges with min and max whiskers. Friedman test with Dunns post-test comparison.

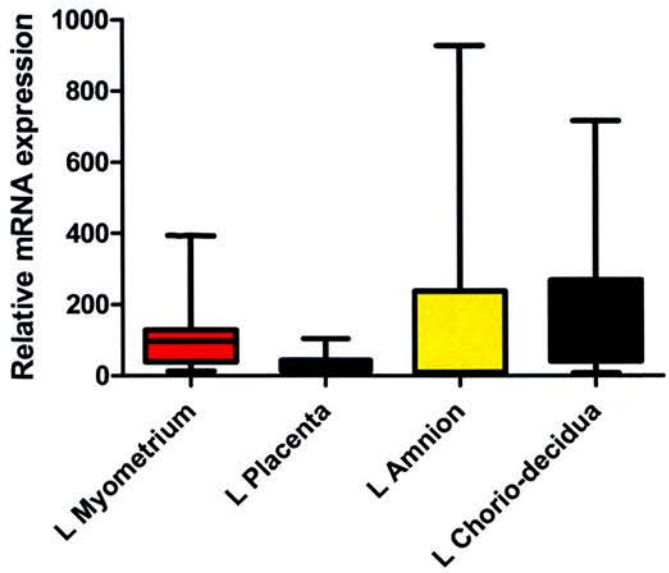


Figure 6.15 FPR1 mRNA expression in labouring tissues

Relative mRNA expression of FPR1 was not significantly different in the four labouring (L) tissues of myometrium, placenta, amnion and chorio-decidua. Data are presented as medians and inter-quartile ranges with min and max whiskers. Friedman test with Dunns post-test comparison.

6.3.4. Relative mRNA expression of 11βHSD1 did not differ in labouring and non-labouring myometrium, placenta, amnion and chorio-decidua

mRNA expression of 11βHSD1 was detected in all of the studied tissues. There were no significant differences in relative mRNA expression of 11βHSD1 in labouring and non-labouring myometrium (Fig 6.16), placenta (Fig 6.17), amnion (Fig 6.18) and chorio-decidua (Fig 6.19).

Relative 11βHSD1 expression was greater in chorio-decidua than in placenta and amnion in both non-labouring and labouring tissues ($p<0.0001$, figs 6.20 and 6.21).

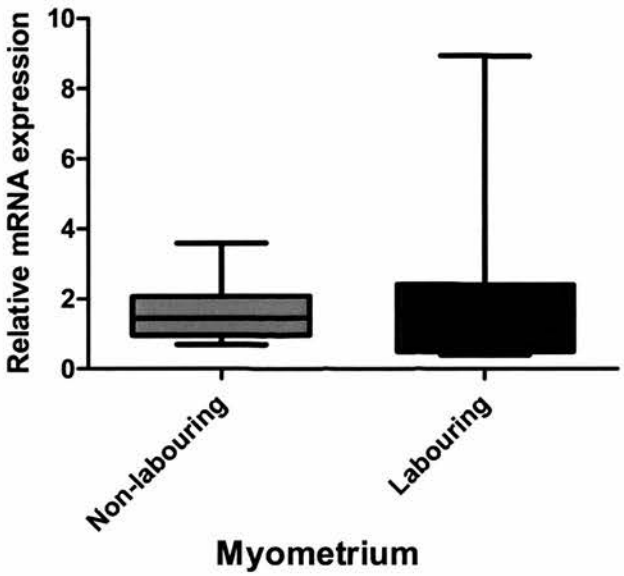


Figure 6.16 11βHSD1 mRNA expression in myometrium

Relative mRNA expression of 11βHSD1 is not significantly different in labouring compared to non-labouring myometrium. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.74, Mann-Whitney test.

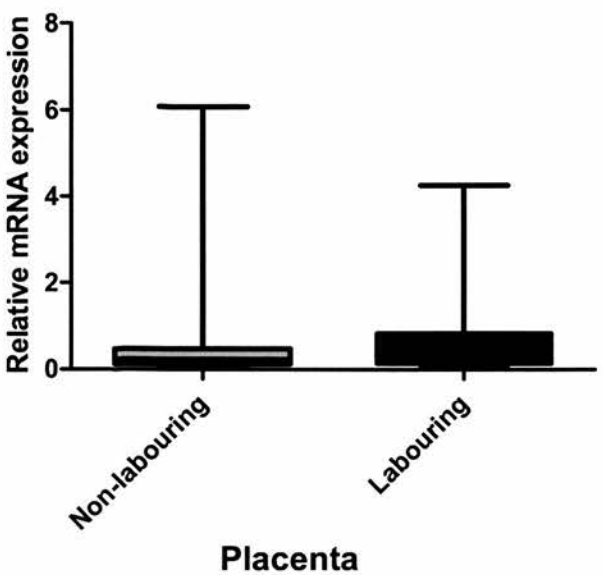


Figure 6.17 11βHSD1 mRNA expression in placenta

Relative mRNA expression of 11βHSD1 is not significantly different in labouring compared to non-labouring placenta. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=1.0, Mann-Whitney test.

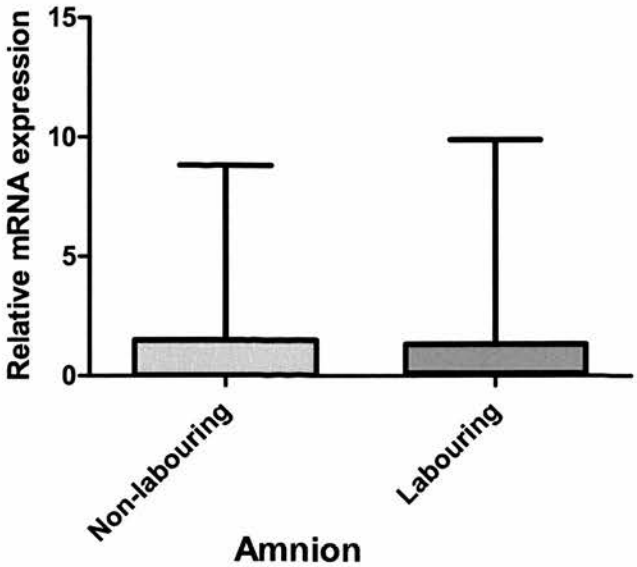


Figure 6.18 11βHSD1 mRNA expression in amnion

Relative mRNA expression of 11βHSD1 is not significantly different in labouring compared to non-labouring amnion. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.22, Mann-Whitney test.

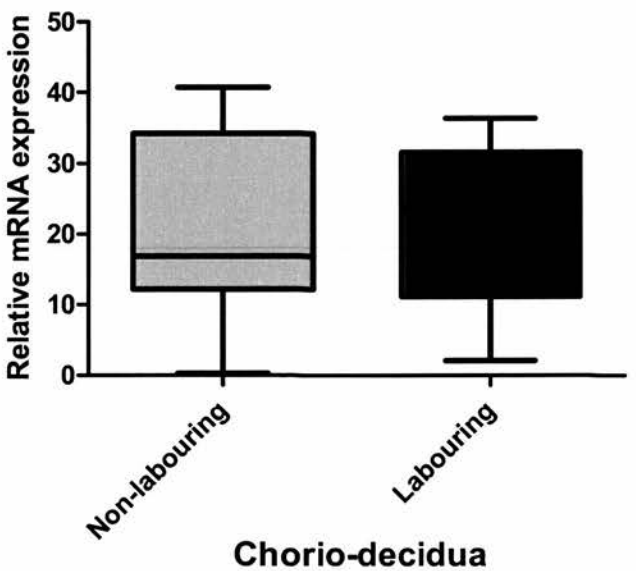


Figure 6.19 11βHSD1 mRNA expression in chorio-decidua

Relative mRNA expression of 11βHSD1 is not significantly different in labouring compared to non-labouring chorio-decidua. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.74, Mann-Whitney test.

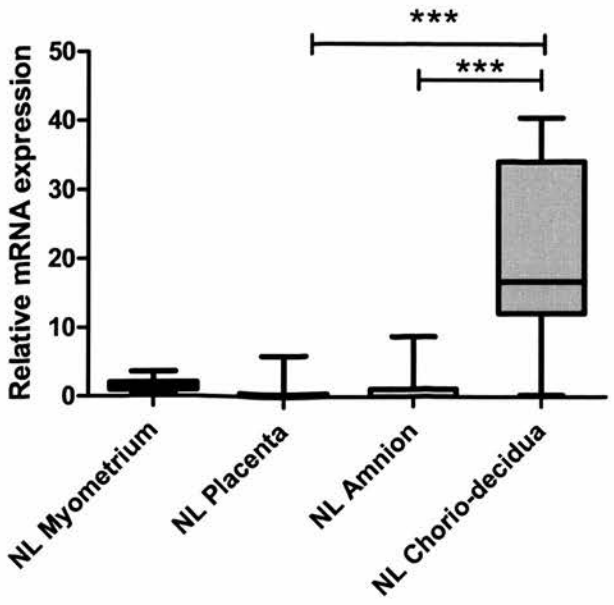


Figure 6.20 11βHSD1 mRNA expression in non-labouring tissues

Relative mRNA expression of 11βHSD1 is greater in non-labouring (NL) chorio-decidua than in placenta and amnion. Data are presented as medians and inter-quartile ranges with min and max whiskers. Friedman test ($p<0.0001$) with Dunns post-test comparison. ***$p<0.001$

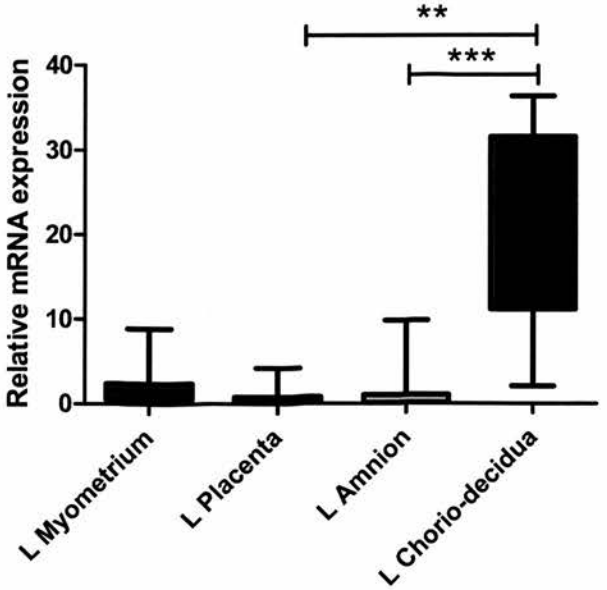


Figure 6.21 11βHSD1 mRNA expression in labouring tissues

Relative mRNA expression of 11βHSD1 is greater in labouring (L) chorio-decidua than in placenta and amnion. Data are presented as medians and inter-quartile ranges with min and max whiskers. Friedman test ($p<0.0001$) with Dunns post-test comparison. ***$p<0.001$

6.3.5. Relative mRNA expression of 11βHSD2 did not differ in labouring and non-labouring myometrium, placenta, amnion and chorio-decidua

mRNA expression of 11βHSD2 was detected in all of the studied tissues. There was no significant difference in relative mRNA expression of 11βHSD2 in labouring and non-labouring myometrium (Fig 6.22), placenta (Fig 6.23), amnion (Fig 6.24) and chorio-decidua (Fig 6.25).

Expression was greater in placenta than in myometrium, amnion and chorio-decidua, in both non-labouring (Fig 6.26) and labouring (Fig 6.27) tissues ($p<0.0001$).

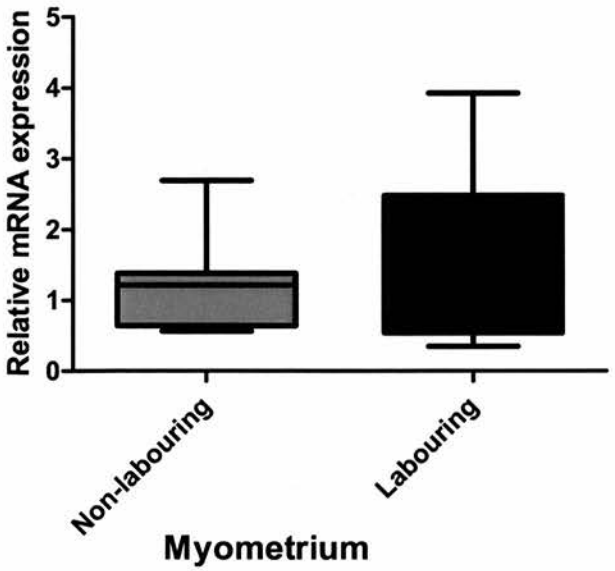


Figure 6.22 11βHSD2 mRNA expression in myometrium

Relative mRNA expression of 11βHSD2 is not significantly different in labouring compared to non-labouring myometrium. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.46, Mann-Whitney test.

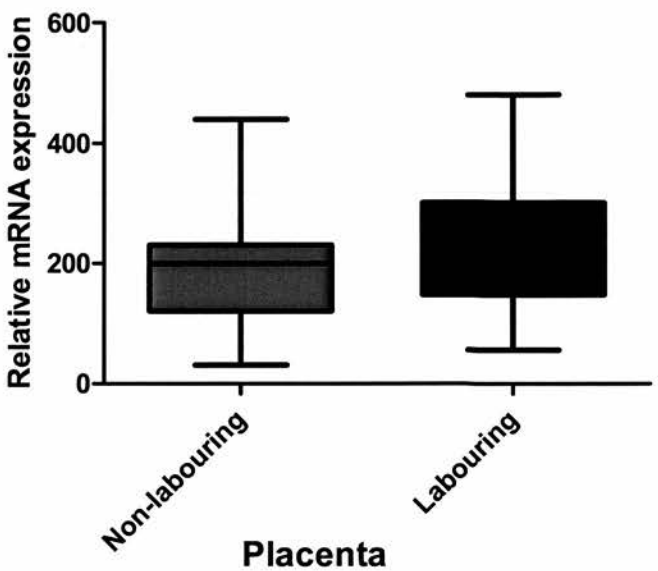


Figure 6.23 11βHSD2 mRNA expression in placenta

Relative mRNA expression of 11βHSD2 is not significantly different in labouring compared to non-labouring placenta. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.42, Mann-Whitney test.

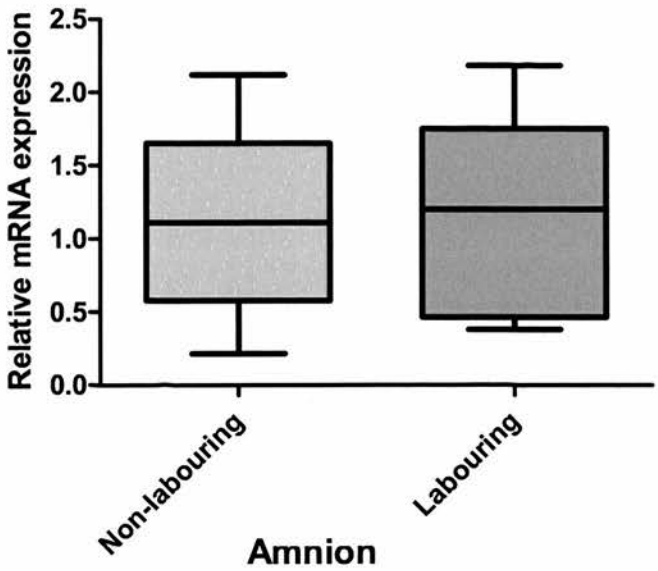


Figure 6.24 11βHSD2 mRNA expression in amnion

Relative mRNA expression of 11βHSD2 is not significantly different in labouring compared to non-labouring amnion. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.69, Mann-Whitney test.

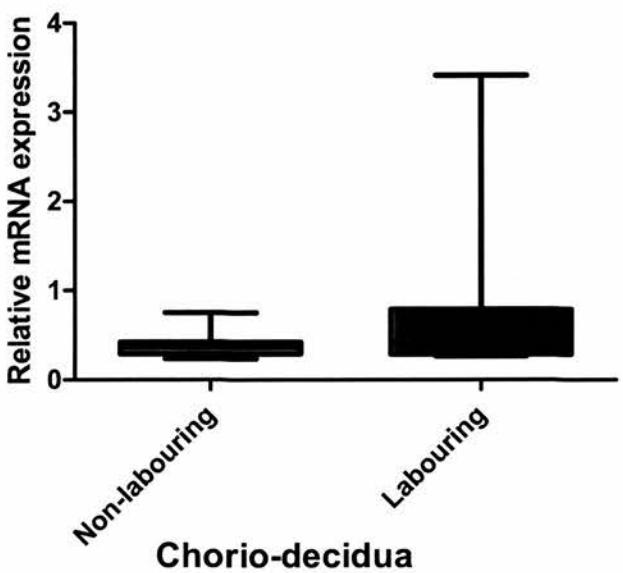


Figure 6.25 11βHSD2 mRNA expression in chorio-decidua

Relative mRNA expression of 11βHSD2 is not significantly different in labouring compared to non-labouring chorio-decidua. Data are presented as medians and inter-quartile ranges with min and max whiskers; n=11 in each group; p=0.36, Mann-Whitney test.

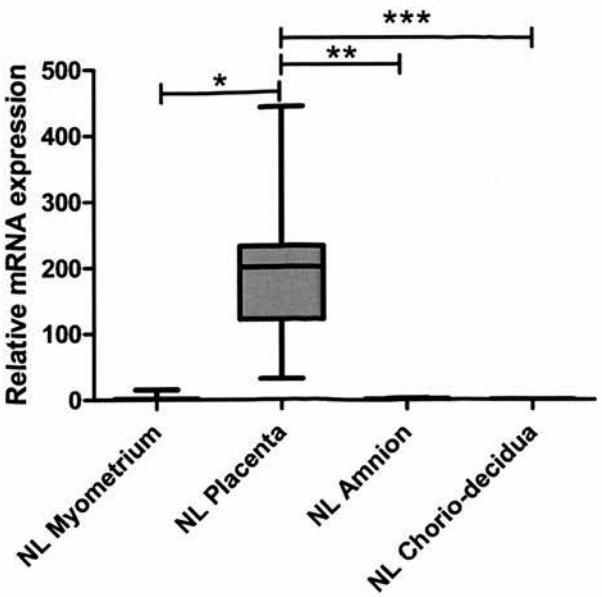


Figure 6.26 11βHSD2 mRNA expression in non-labouring tissues

Relative mRNA expression of 11βHSD2 is greater in non-labouring (NL) placenta than in myometrium, amnion and chorio-decidua. Data are presented as medians and inter-quartile ranges with min and max whiskers. Friedman test ($p<0.0001$) with Dunns post-test comparison. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

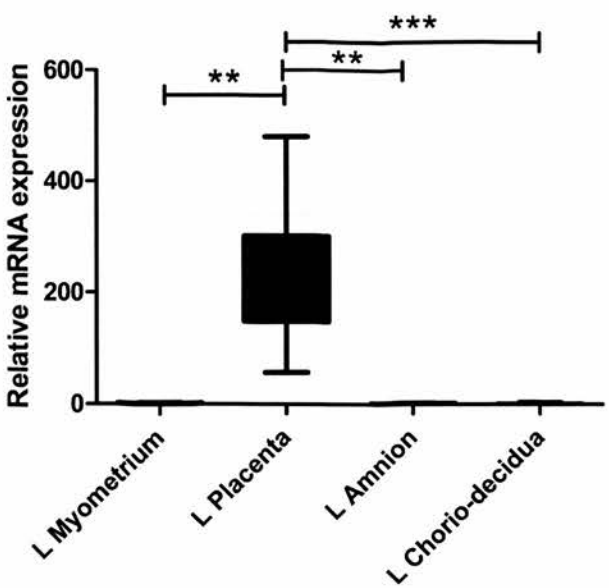


Figure 6.27 11βHSD2 mRNA expression in labouring tissues

Relative mRNA expression of 11βHSD2 is greater in labouring (L) chorio-decidua than in placenta and amnion. Data are presented as medians and inter-quartile ranges with min and max whiskers. Friedman test ($p<0.0001$) with Dunns post-test comparison. ***$p<0.001$, **$p<0.01$

6.3.6. Treatment of myometrial explants with cortisol for 6 or 24 hours did not significantly alter relative mRNA expression of ANXA1 compared to vehicle

There was no significant difference in relative mRNA expression of ANXA1 in myometrial explants treated for 6 hours with cortisol, at concentrations from 10^{-9}M to 10^{-6}M, or with vehicle, (Fig 6.28).

Treatment with the highest dose of cortisol (10^{-6}M) for 24 hours also had no effect on relative mRNA expression of ANXA1 compared with tissues treated with vehicle (Fig 6.29).

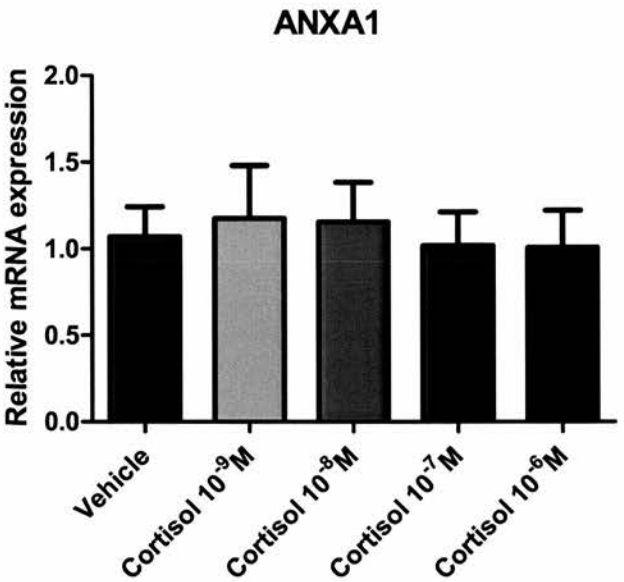


Figure 6.28 ANXA1 mRNA expression in myometrial explants, 6 hour culture

Relative mRNA expression of ANXA1 was not significantly different in myometrial explants treated with cortisol (range of concentrations 10^{-9} to 10^{-6}M) compared to vehicle. Myometrial explants treated for 6-8 hours at 37°C in room air. Data are presented as means +SEM; n=5; p=0.18, Friedman test with Dunn's multiple comparison test.

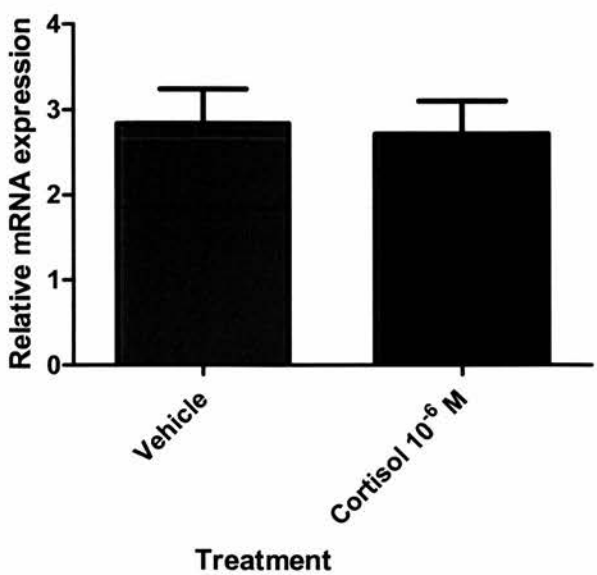


Figure 6.29 ANXA1 mRNA expression in myometrial explants, 24 hour culture

Relative mRNA expression of ANXA1 was not significantly different in myometrial explants treated with 10^{-6}M cortisol for 24 hours compared to vehicle. Myometrial explants treated for 24 hours at 37°C in room air. Data are presented as means +SEM; n=5, paired t-test, p=0.81.

6.3.7. Treatment of myometrial explants with cortisol for 6 hours upregulated relative mRNA expression of both FPR1 and FPR2/ALX

Treatment with cortisol at a concentration of 10^{-6}M for 6 hours upregulated the mRNA expression of FPR2/ALX in myometrial explants compared to treatment with vehicle (Fig 6.30) (n=5, Friedman test, p=0.0028, with Dunn's multiple comparison test, $p<0.05$, relative mean fold change 16.5). Statistically significant different relative levels were also observed when comparing the lowest dose of 10^{-9}M with the highest dose of 10^{-6}M (n=5, Friedman test, p=0.0028, with Dunn's multiple comparison test, $p<0.05$, relative mean fold change 15.3). At lower doses, ranging from 10^{-9}M to 10^{-7}M, there was no significant difference in relative expression between cortisol-treated explants from those treated with vehicle.

There was a trend towards increased relative mRNA expression of FPR2/ALX (Fig 6.31) in myometrial explants treated with 10^{-6}M cortisol for 24 hours compared with treatment with vehicle. However, this did not quite reach statistical significance (n=5, paired t-test, p=0.07).

Since the effect of cortisol on FRP2/ALX expression was only observed at 10^{-6}M concentration, I subsequently investigated the effect of this dose of cortisol on FPR1 mRNA expression. Treatment with 10^{-6}M cortisol for 6 hours upregulated the mRNA expression of FPR1 in myometrial explants compared with treatment with vehicle (Fig 6.32) (n=5, paired t-test, P=0.04, relative mean fold change 7.4). There was a trend towards increased relative mRNA expression of FPR1 (Fig 6.33) in myometrial explants treated with 10^{-6}M cortisol for 24 hours compared with treatment with vehicle. However, this did not quite reach statistical significance (n=5, paired t-test, p=0.06).

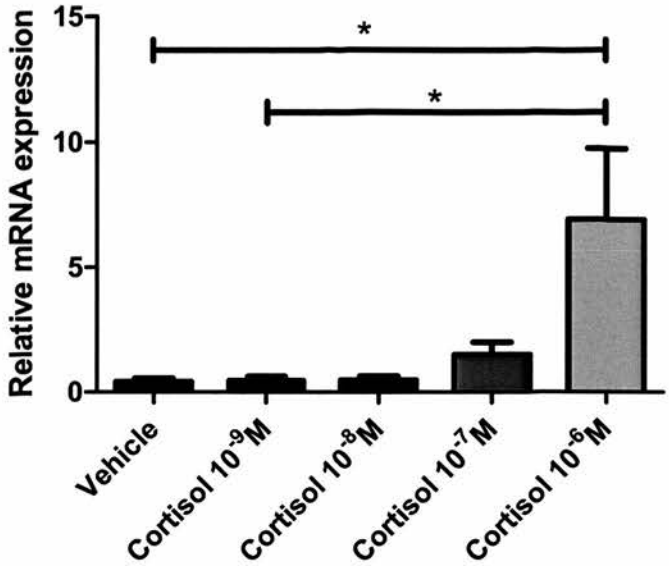


Figure 6.30 FPR2/ALX mRNA expression in myometrial explants, 6 hr culture

Relative mRNA expression of FPR2/ALX was greater in myometrial explants treated with cortisol at a dose of 10^{-6}M for 6 hours than in explants treated with vehicle (relative mean fold change 16.5). Treatments of cortisol at a dose of 10^{-7}M or lower did not result in significantly different expression levels. Explants were cultured for 6-8 hours at 37°C in room air. Data are presented as means +SEM; n=5; p=0.0028, Friedman test with Dunn's multiple comparison test, *p<0.05.

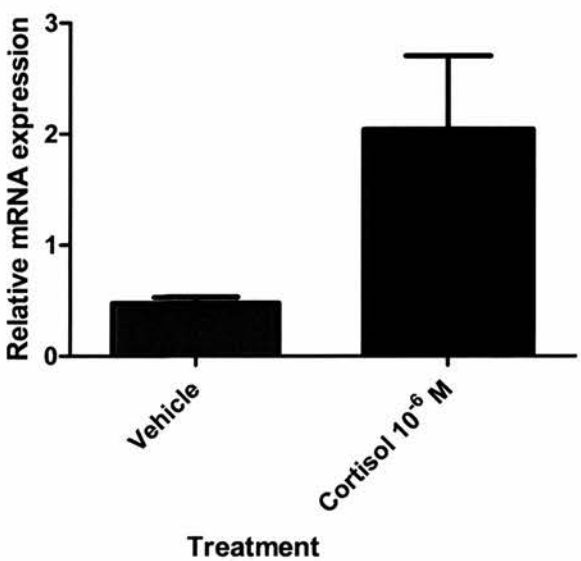


Figure 6.31 FPR2/ALX mRNA expression in myometrial explants, 24 hr culture

Relative mRNA expression of FPR2/ALX was not significantly different in myometrial explants treated with 10-6M cortisol for 24 hours compared to treatment with vehicle. Myometrial explants were treated for 24 hours at 37°C in room air. Data are presented as means +SEM; n=5, paired t-test, p=0.07.

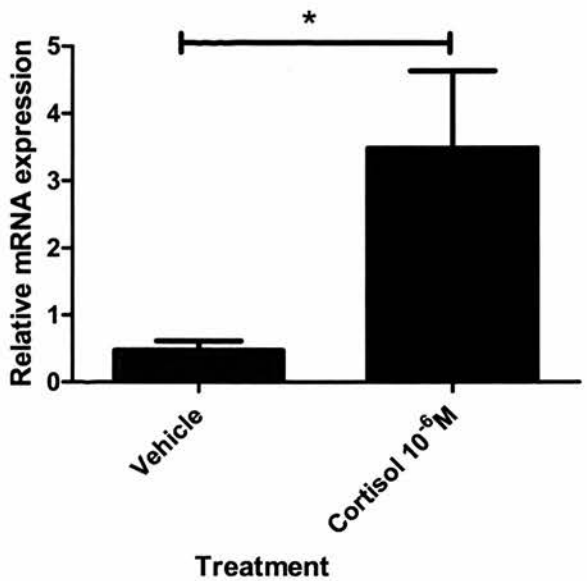


Figure 6.32 FPR1 mRNA expression in myometrial explants, 6 hour culture

Relative mRNA expression of FPR1 was greater in myometrial explants treated with cortisol at a dose of 10^{-6}M than in explants treated with vehicle for 6 hours (relative mean fold change 7.1). Explants were cultured for 24 hours at 37°C in room air. Data are presented as means +SEM; n=5; p=0.04, paired t-test.

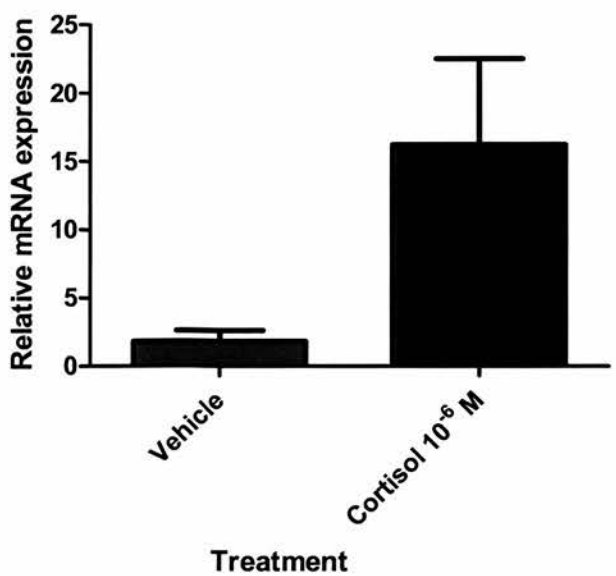


Figure 6.33 FPR1 mRNA expression in myometrial explants, 24 hour culture

Relative mRNA expression of FPR1 was not significantly different in myometrial explants treated with cortisol 10^{-6}M compared to vehicle, when treated for 24 hours. Myometrial explants treated for 24 hours at 37°C in room air. Data are presented as means +SEM; n=5, paired t-test, p=0.06.

6.4. DISCUSSION

The work in this chapter demonstrates that:

- mRNA and protein expression of ANXA1 was detectable in human term pregnant myometrium, placenta, amnion and chorio-decidua.
- Immunohistochemistry localised the ANXA1 protein to myocytes, endothelial cells and neutrophils in myometrium, and to syncytiotrophoblasts and endothelial cells in placenta.
- There was no significant difference in relative expression of ANXA1 mRNA in labouring myometrium, placenta, amnion or chorio-decidua than in non-labouring samples.
- mRNA expression of the receptor FPR1 was also detectable in myometrium, placenta, amnion and chorio-decidua and relative mRNA levels were increased in labouring tissue when compared to non-labouring tissue. This finding is in concert with the observation made in Chapter 3 that the FPR2/ALX receptor was upregulated in labouring tissues.
- 11βHSD1 and 11βHSD2 mRNA was expressed in human term pregnant myometrium, placenta, amnion and chorio-decidua, and there was no significant difference in relative expression of these genes in labouring myometrium, placenta, amnion or chorio-decidua than in non-labouring samples.
- Treatment of myometrial explants with cortisol for 6 hours did not significantly alter relative mRNA expression of ANXA1 compared to vehicle.
- Treatment of myometrial explants with cortisol for 6 hours upregulated relative mRNA expression of both FPR1 and FPR2/ALX.

In myometrium, I showed that ANXA1 localised to myocytes, neutrophils and endothelial cells surrounding the blood vessels. Strongest staining was seen in the neutrophils and vascular endothelial cells. This pattern of staining was observed in

both labouring and non-labouring tissues, although in labouring tissues there were increased numbers of invading neutrophils.

In placenta, I showed that ANXA1 localised to the syncytiotrophoblasts, with the strongest staining in the brush border contacting the maternal circulation. Staining was also observed in the cells surrounding the blood vessels and in neutrophils. Similar findings were reported by Sun et al, who also reported no observable difference in distribution between labouring and non-labouring tissues (Sun et al. 1996).

In fetal membranes, ANXA1 localised to the amnionic epithelial cell layer, to the chorionic mesoderm and to trophoblastic cells. The immunohistochemical techniques reported here do not allow comment on changes in concentration or expression levels of the protein or its RNA; only on distribution, but expression levels of mRNA determined by quantitative RT-PCR are discussed below.

In this study, relative expression of ANXA1 mRNA was not different in labouring myometrium, placenta, amnion or chorio-decidua compared to non-labouring tissues of the same kind. Previous investigators have reported different findings regarding expression of ANXA1 in the placenta and fetal membranes, as I shall now discuss.

Myatt's group used Northern and Western blots to quantify mRNA and protein levels in amnion (Lynch-Salamon et al. 1992), and protein levels in chorion (Myatt et al. 1992). They found that levels of ANXA1 mRNA were significantly reduced in amnion after labour, and that protein levels were correspondingly reduced in both amnion and chorion following labour. However, Bennett et al, again using Northern blotting, reported a decrease in total RNA expression of ANXA1 in labouring amnion and placenta, but an increase in labouring chorio-decidua (Bennett et al. 1994). They also looked at annexin 2, which was increased only in labouring amnion and at phospholipase A2 and annexin 5, both of which demonstrated no difference in the labouring and non-labouring tissues. There are therefore some contradictions between these data, and between them and the results in my study. It may be that

discrepancy between this published work and my study reflects differences encountered when using the older technique of Northern blotting to look at RNA expression, whereas my study has used the more sensitive technique of quantitative RT-PCR.

There was also a difference in the timings of when samples were collected in the previously reported studies. Both Bennett's and Myatt's studies compared placentae taken at elective caesarean section (pre-labour) and placentae delivered vaginally (post-labour) whereas our study compares placentae taken at elective caesarean section (pre-labour) or at emergency caesarean section (during labour). Thus we have compared pre-labour tissue with tissue in the second stage of labour, whereas the other work compare pre-labour tissue with tissue sampled after completion of the third stage of labour (Lynch-Salamon et al. 1992; Myatt et al. 1992; Bennett et al. 1994). Clearly the function of the placenta and membranes varies greatly during the second and third stage of labour. During the second stage the placenta is still adherent to the uterine wall and is exchanging oxygen, CO_2 and other metabolites between the maternal and fetal circulations. During the third stage the fetus is no longer dependent on the placenta for oxygen and is indeed often detached from the placenta, and the placenta detaches from the uterine wall and is expelled. The function of the uterus also changes dramatically at completion of the third stage. It is then required to expel the placenta and membranes and change from producing regular contractions to achieving a tonic contraction which involutes the organ, constricts spiral vessels and limits postpartum haemorrhage. It may be therefore that the differences in the reported results actually reflect differences in the expression of ANXA1 between the second stage and completion of the third stage. Given that the previously published work has used different laboratory techniques to this work, it would be interesting to use our quantitative RT-PCR technique to compare vaginally delivered tissue with the current groups already studied.

In addition to their findings in RNA, Lynch-Salamon et al also reported a reduction in protein levels of ANXA1 in post-delivery amnion when compared to pre-labour amnion (Lynch-Salamon et al. 1992). My study has not examined protein levels of

ANXA1, although this would be an interesting subsequent study, as it may be that mRNA levels are not necessarily representative of the protein levels. Furthermore, levels of protein do not necessarily reflect activation status of the protein i.e. the tissue may contain protein that has not been mobilised to the cell surface and is currently not available to bind to the receptor.

PMNs contain high levels of ANXA1 (Perretti et al. 2000), with an estimated 2-4% protein content represented by ANXA1. A large proportion of this is contained in granules or vesicles (Perretti et al. 1996; Perretti et al. 2000) and upon activation and adhesion of the PMN the protein is mobilised to the membrane compartment and the cell surface (Perretti et al. 2000). Thus, given that parturition is heralded by an influx of neutrophils into myometrium (Thomson et al. 1999), it could be expected that increased levels of ANXA1 might have been observed. Although in the immunolocalisation studies presented here the strongest staining was to be seen in PMNs, there was also staining throughout the myocytes. Even if increased numbers of neutrophils did produce increased amounts of ANXA1, it may be that the background level of ANXA1 represented by production from myocytes was sufficient to mask any increase represented by the invading neutrophils. The data presented in this chapter looks at the expression of mRNA levels of ANXA1, and this may not be representative of either protein levels or indeed active levels of protein.

Ultimately therefore, whilst it is interesting that labour does not appear to upregulate de novo synthesis of ANXA1 in reproductive tissues, further work is necessary to clarify what actual levels of active and available ANXA1 protein may be present either in circulating blood, or locally in the reproductive tissues in labour. Currently within our laboratory, work is underway to develop an assay to determine ANXA1 levels, and this will no doubt produce interesting future data. Given the discussion regarding location of ANXA1 expression and whether or not any increase in levels may be contributed by myocytes, or whether expression is overwhelmingly neutrophilic in origin, fluorescence-activated cell sorting (FACS) may help in answering this question. FACS is a technique by which different types of cell are

sorted based upon their individual fluorescent and light scattering characteristics. Thus a sample of tissue could be analysed by FACS using markers to identify the cell types, for example, myocytes and neutrophils, and other fluorescent markers to demonstrate the presence or absence of ANXA1 within the different cell groups. Labouring and non-labouring tissues could then be compared to determine in which cell type, if any, differences in ANXA1 protein expression could be observed. Again, this work was outwith the scope of this project, but will be interesting in future work.

As reported in Chapter 3 of this thesis, the FPR2/ALX receptor is upregulated in human labour. Thus, ANXA1 may have a role in labour via increased availability of its receptor, even in the absence of increased levels of the ligand. Whilst full-length ANXA1 has been demonstrated to signal exclusively through FPR2/ALX (Walther et al. 2000; Hayhoe et al. 2006), the short peptides which are cleavage products of ANXA1 signal through both FPR2/ALX and the closely related receptor FPR1 (Hayhoe et al. 2006). Thus, we also looked at mRNA expression levels of FPR1 in the same samples as those which had demonstrated an increase in FPR2/ALX mRNA in labour, to see if this finding was matched for FPR1.

FPR1 mRNA expression was upregulated in labour in myometrium, placenta, amnion and chorio-decidua with median fold changes of 10.9, 3, 4.3 and 26.2 respectively. This finding mirrors the finding discussed in Chapter 3 in which the FPR2/ALX receptor is also upregulated in the same samples, demonstrating an upregulation of the whole system. FPR1 and FPR2/ALX are closely related and both have been demonstrated to be upregulated by glucocorticoids (Sawmynaden and Perretti 2006; Ehrchen et al. 2007), thus it is interesting to note a similar response by both receptors to labour. Although we have not demonstrated evidence that there are increased levels of ANXA1 in labour, there is certainly upregulation of both of the receptors through which it and its cleavage peptides signal, thus providing a potential mechanism by which its effects may be amplified.

Both FPR1 and FPR2/ALX are promiscuous receptors and have a number of different reported ligands, both agonists and antagonists, in addition to LXA4 and

ANXA1 (Ye et al. 2009), which have been studied in this work. Indeed, although both LXA4 and ANXA1 induce anti-inflammatory and pro-resolution effects when signalling through FPR2/ALX, there are also ligands which induce pro-inflammatory effects (Ye et al. 2009). For example, the acute-phase protein serum amyloid A (SAA) has been reported in neutrophils to induce the secretion of IL-8 by signalling through FPR2/ALX (He et al. 2003). It could be that a number of ligands compete for binding of FPR2/ALX during parturition and the effect of FPR2/ALX ligand interaction will be crucially dependent on the nature of that ligand.

6.4.1. The effect of cortisol on the ANXA1-FPR1/2 system

The data in this thesis have demonstrated detectable mRNA levels of both 11βHSD1 and 11βHSD2 in myometrium, placenta, amnion and chorio-decidua. 11βHSD1 was most highly expressed in chorio-decidua, whilst 11βHSD2 was most highly expressed in placental tissue. When comparing labouring tissue with non-labouring tissue of the same type, there were no significant differences seen. High levels of placental 11βHSD2 are well documented in the literature, and it is thought this is a protective measure to convert cortisol to the inactive cortisone to reduce exposure of the fetus to high levels of maternal cortisol, thus shielding it from its potentially deleterious effects. Similarly, it has been shown that the fetal membranes express high levels of 11βHSD1 and the theory has been put forward that the subsequent local control of cortisol levels may be involved in the process of parturition.

11βHSD1 protein has previously been shown to be found in placenta (Pepe et al. 1999), amnion and chorio-decidua (Sun et al. 1997; Pepe et al. 1999; Alfaidy et al. 2003) using immunohistochemical techniques and western blotting, with western blotting demonstrating higher levels of protein in the amnion and chorion than in the placenta (Alfaidy et al. 2003). Sun et al observed no differences in strength of immunohistochemical staining between labouring and non-labouring placenta, amnion and chorion (Sun et al. 1997), but later work from the same group found that Western analysis found more 11βHSD1 in labouring amnion and chorion than in their non-labouring counterparts, although this was only significant in amnion (Alfaidy et al. 2003). 11βHSD1 increases in both protein levels and activity in fetal

membranes with advancing gestation, but there is no gestational-age related change in placenta (Alfaidy et al. 2003). The work reported from my study is broadly in agreement with previously published work in that mRNA for 11βHSD1 has been demonstrated to be found in placenta, amnion and chorio-decidua, with much higher levels in chorio-decidua than in placenta. In contrast, my samples demonstrated mRNA levels in amnion to be more similar to those in placenta than in chorio-decidua, whereas other reports describe similar levels in amnion and chorion (Alfaidy et al. 2003). This may be a reflection of the different analyses (western vs quantitative RT-PCR), or it may be a reflection of the variation in individual samples. Our samples showed large variation in mRNA expression between individual samples in the fetal membranes, and this is also observed by other researchers (Sun et al. 1997). The fetal membranes are acknowledged to have widely varying enzyme expression and activity in differing areas of the same membrane (El Khwad et al. 2005). This may be particularly relevant when considering their production of PGDH, the enzyme that degrades prostaglandins (Cheung et al. 1990), because prostaglandins also modulate 11βHSD1 levels (Alfaidy et al. 2003). The work presented here has focused on mRNA expression and attempted to explore any differences between labouring and non-labouring tissues. In concordance with most of the published data on protein levels, I observed no differences between labouring and non-labouring 11βHSD1. Thus, it would appear that although 11βHSD1 increases towards term (Alfaidy et al. 2003), which may have an influence on local cortisol production towards the end of gestation, this influence is not acutely altered during labour itself. Little data is published on the expression of 11βHSD1 in myometrium, and my data has shown that there is indeed mRNA expression of this enzyme in both labouring and non-labouring myometrium, although, as for the other studied tissues, no difference in parturition is seen. However, expression levels of 11βHSD1 mRNA in myometrium were low in comparison to that seen in chorio-decidua, being more similar to those observed in amnion and placenta, suggesting therefore that myometrium is a much less important site for local control of cortisol levels than the fetal membranes.

11βHSD2 is found in abundance in placenta (Krozowski et al. 1995; Sun et al. 1997; Pepe et al. 1999), and this finding was also true in my samples, with significantly larger expression in placenta than in any other tissues. Sun et al detected 11βHSD2 mRNA by Northern blot analysis in placenta, but not in amnion or chorion (Sun et al. 1997), therefore my work showing its presence in amnion, chorio-decidua and myometrium, is contrary to that. Certainly the levels were much lower than those shown in placenta and it is likely that the main site of action is indeed in the placenta.

The differential distribution of the 11βHSD enzymes in the feto-maternal unit in term pregnancy are likely to provide a mechanism by which local concentrations of cortisol may be controlled. Relevant functional consequences already described include shielding of the fetus from excess glucocorticoid and potential influences on prostaglandin production during parturition, and a further consequence may be the effect that altered cortisol levels may have on ANXA1, its production, release and activity during pregnancy and parturition.

In the study reported here, cortisol did not affect ANXA1 mRNA expression in myometrial explants, a finding which is supported by the observation that ANXA1 mRNA was not upregulated in labour, a state in which cortisol levels are increased. Zhang et al report that whilst cortisol upregulates ANXA1, as determined by immunoblotting, the upregulation is weak in comparison to the upregulation seen with more potent glucocorticoids such as betamethasone and dexamethasone (Zhang et al. 2010). It would be interesting to repeat my experiment with other glucocorticoids, particularly since both betamethasone and dexamethasone are used clinically in administration to mothers in threatened preterm labour to aid maturation of the fetal lung. Despite there being no change in measurable mRNA levels of ANXA1 in myometrial explants with cortisol treatment, mRNA levels of both receptors, FPR1 and FPR2/ALX were significantly upregulated by treatment with cortisol. Again, this mirrors the upregulation demonstrated in labouring tissues and may provide a mechanism by which the effects of ANXA1 may be amplified.

6.4.2. Conclusions

A diagrammatic representation of the interactions between cortisol, the FPRs and ANXA1 and its N-terminal peptides is illustrated in Figure 6.34.

ANXA1 mRNA expression is unchanged in myometrium, placenta, amnion and chorio-decidua in labour, although the mRNA expression of the receptors FPR1 and FPR2/ALX are increased. Thus there is a potential role for ANXA1 in the process of parturition via increased expression of the receptors and thus increased activity of ANXA1.

In other systems, the main described functions of ANXA1 cssentially focus on an effect on neutrophil trafficking, by reducing neutrophilic invasion into a site of inflammation (Perretti et al. 1996), by promoting their apoptosis (Solito et al. 2003) and promoting clearance of apoptotic cells by macrophages (Scannell et al. 2007). In labour, there is an influx of neutrophils into the myometrium and cervix that produce pro-inflammatory cytokines, which are felt to be one of the driving forces of the complex process of parturition. To what end would it be beneficial for this process to be opposed? It may be that the role of ANXA1 lies within the resolution of this inflammatory process; in ensuring that when labour is over the uterus is able to return to its pre-labour and pre-pregnancy state. Alternatively ANXA1 may have a role during pregnancy itself in ensuring that mild inflammatory insults are neutralised and resolved without escalating into an inflammatory process that results in labour. Further work exploring ANXA1, its N-terminal peptides and FPR1&2 levels during pregnancy and in pre-term labour may help provide clues as to whether this theory is valid. It would also be interesting to expose ANXA1 null mice to LPS and observe what effect this has. Researchers in our laboratory investigating fetal white matter damage use a rat model in which a mild insult of LPS is administered not sufficient to initiate preterm labour, but which results in lower birth weight pups and possible white matter injury (personal communication, Dr E Pilley, Clinical Research Fellow, University of Edinburgh). Similar mouse models have been shown to alter gene expression in the brains of exposed offspring (Elovitz et al. 2011). Such a model could be used to compare ANXA1 null animals with wild type animals with

the hypothesis that ANXA1 null animals would be more likely to labour prematurely and more likely to have poorer observable outcomes for the offspring.

Ultimately, there is much more that needs to be discovered about the potential role of ANXA1 in pregnancy and parturition. It seems likely that in the future, therapeutic agents for preterm labour, will take a multitude of approaches and that anti-inflammatory agents may well a vital part of that approach, both to moderate the inflammatory process that has been initiated and to protect the fetal brain from the harmful environment of antenatal infection and inflammation.

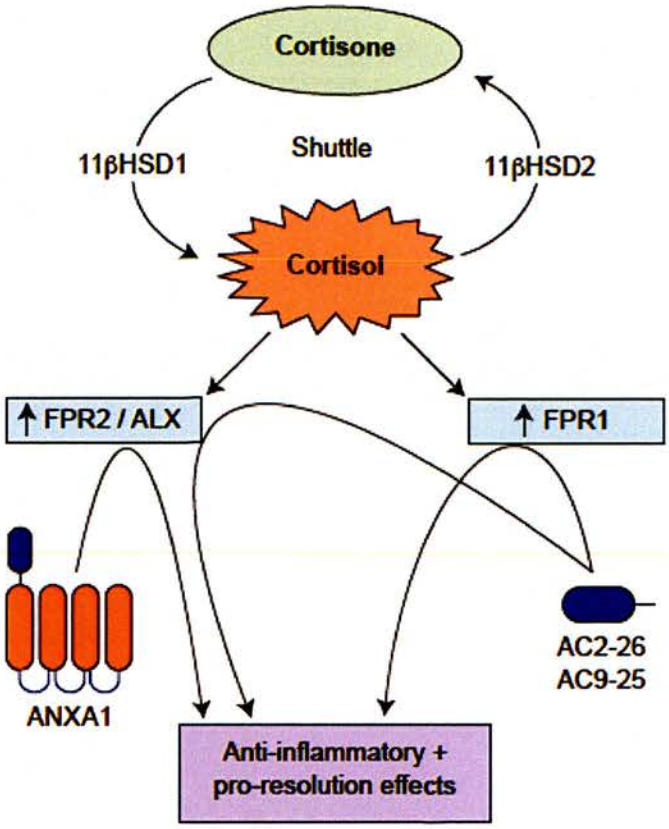


Figure 6.34

Diagrammatic representation of the interactions between the cortisone-cortisol shuttle, the formyl peptide receptors and ANXA1 and its N-terminal fragment peptides.

The production of the active cortisol from its inactive form cortisone is controlled by the 11βHSD enzymes in the cortisone-cortisol shuttle. There is likely to be control of local cortisol levels within the feto-placental unit by these enzymes. Cortisol upregulates FPR2/ALX and FPR1, through which ANXA1 and its N-terminal fragment peptides signal. Increased availability of the receptors may provide a mechanism by which they have greater effects.

7. General Discussion

7.1. SUMMARY OF FINDINGS

Preterm and dysfunctional term labour present a significant and ongoing burden to the health of women and infants worldwide, and we currently lack truly effective interventions to treat and control these conditions. Human labour is an inflammatory condition and so better understanding of the inflammatory processes that initiate and promote term and preterm labour are vital to the development of new therapies to treat abnormal labour. This thesis examined the role of pro-resolution mediators in human pregnancy and parturition, specifically lipoxin A4 and Annexin A1, both of which signal through the same receptor, FPR2/ALX. The main findings of this work are summarised below.

- Circulating levels of LXA4 are increased in pregnancy compared to non-pregnancy, increasing to term, after which there is no further increase (Chapter 3).
- mRNA expression of FPR2/ALX is greater in the reproductive tissues of the during labour compared to pre-labour (Chapter 3).
- LPS evokes an inflammatory response in pregnant myometrium that has similarities to the inflammatory signature of parturition (Chapter 4).
- Hypoxic culture of myometrial explants has no effect on myometrial production of LXA4 , but possibly may influence expression of FPR2/ALX (Chapter 5).
- mRNA expression of ANXA1 is not different in labour compared to non-labour in reproductive tissues and is not affected by cortisol treatment in a myometrial explant model (Chapter 6).
- Cortisol treatment upregulates myometrial FPR2/ALX and FPR1 expression (Chapter 6).

7.2. LXA4

The pro-resolution effects of LXA4 are relatively recently described and much is yet to be learnt about its effects in the reproductive system. It is highly likely that LXA4 has a role within pregnancy because circulating levels are higher in pregnancy and remain so right up until delivery. Pregnant women are in a state of immunosuppression in order to allow tolerance of the fetal allograft. They are therefore at increased risk of infection, which presents an increased risk of preterm labour. Even if infection is insufficient to cause preterm labour, increasing evidence is emerging that an inflammatory state can cause significant permanent damage to the developing fetus, even when the inflammatory response has been insufficient to cause preterm labour (Elovitz et al. 2011). Thus a delicate balance must be struck by the immune system, to provide vigilant suppression of any invading pathogen and inflammatory insult to protect the mother and reduce the risk of preterm labour, whilst at the same time, not responding so avidly to infection or inflammation that preterm labour initiates inappropriately or the fetus is exposed to an excessively inflammatory and damaging environment. It is possible that the role of both LXA4 and ANXA1 within pregnancy is that of a 'checkpoint' and that increased levels of LXA4 in pregnancy are required to act as a braking signal for inappropriate inflammation.

LXA4 levels are not increased during labour, and the data presented in this thesis suggest that levels may even fall in the days preceding labour, which would fit with the concept of the brake being eased off and the cascade of inflammation being allowed to proceed and labour to initiate. The upstream signalling pathways that might provoke increased production of LXA4 in pregnancy and potentially contribute to any reduction as the 'brake' is eased off towards delivery are unknown. Further work to elucidate the nature of such pathways may provide important clues towards what initiates labour itself. As discussed in the Introductory chapter of this thesis, although labour is primarily an inflammatory process, there are complex interactions with other systems, particularly the endocrine system. Estrogen, progesterone and CRH in particular have intricate crosstalk with the immune system and it would be very interesting to examine their interactions with pro-resolution

mediators. Functional genomics are an exciting way in which many different aspects of signalling and function of a process can be explored and it was somewhat disappointing that the microarray performed in the work for this thesis was unable to give any answers regarding potential pathways involving LXA4 due to the contamination of the LXA4 used. Certainly the pathways displayed by treating with LPS provide insights to the type of inflammatory pathways that may prove to be worthy targets for reducing inappropriate inflammation in myometrial tissue in pregnancy. Future work may include repeating a similar experiment with an alternative source of LXA4 that has been demonstrated to have functional effects in myometrium.

Most of the work in this thesis has focused on working with samples gathered from women at term in their pregnancy. Term and preterm labour are of course similar processes, but they are not identical. However, an understanding of the processes of term labour is important to also understand why it is that some pregnancies do not proceed to term. The work presented here has produced some data from which it would be interesting to extrapolate to samples collected from women either at risk of preterm labour or actually in preterm labour. Specifically, future work may include examining longitudinal samples taken from women during pregnancy who are at low or high risk of preterm labour, looking at levels of pro-resolution mediators and the expression of the relative receptors. Inevitably in any population of women recruited in early pregnancy, some women would labour preterm, and more information about pro-resolution mediators in this process could provide ways of developing either a way of predicting women at increased risk of preterm labour, or even better ways of preventing preterm labour.

7.3. ANXA1

ANXA1 expression does not appear to increase during labour, as is the case with LXA4. However, recent published data have demonstrated that, also like LXA4, expression is increased in pregnancy when compared to non-pregnancy (Macdonald et al. 2011). Again, it may be that ANXA1 provides a mechanism of restraint on pregnancy, to allow quiescence of the feto-placental unit and to allow the fetus to grow and develop until it is sufficiently mature to meet the outside world. FPR2/ALX expression is increased in labour, as is the expression of FPR1 through which short peptides related to ANXA1 signal. Thus even though expression of ANXA1 appears to be unchanged in parturition, there is the potential for them to have increased effect via increased expression of the receptor. However, functional effects of LXA4 or ANXA1 due to the increased FPR2/ALX have not been demonstrated. FPR2/ALX and FPR1 are both promiscuous receptors and both have the ability to bind with both pro- and anti-inflammatory ligands (Ye et al. 2009). For example, FPR2/ALX binds with serum amyloid A, an acute phase protein which has powerful pro-inflammatory effects. It is not known to what extent FPR2/ALX has preferential binding toward pro- or anti-inflammatory ligands in the reproductive system or whether this changes during pregnancy or parturition. It may be that an increase in expression of the receptor allows increased anti-inflammatory or pro-resolution effects despite unchanged absolute levels of the pro-resolution ligands, or it may be that as the overwhelming inflammatory cascade of labour commences, LXA4 and ANXA1 have a less and less significant effect and increased levels of the receptor serve actually to promote the effects of pro-inflammatory ligands. Colleagues in our laboratory have begun to explore the effects of pro-inflammatory ligands of FPR2/ALX in the reproductive system and future work may include examining competition for binding at the receptor by the different ligands, whether or not this is important in parturition, and if so, whether or not this can be manipulated to the advantage of controlling the onset of labour.

ANXA1 is known already to be affected by glucocorticoids, and although levels were not affected in the explant experiment reported in this thesis, levels of both FPR2/ALX and FPR1 were, thus providing a mechanism by which ANXA1 may

have an altered effect. The glucocorticoid used was cortisol, an endogenous steroid, although exogenously administered steroids such as betamethasone and dexamethasone have been reported to have much more potent effects on ANXA1 than cortisol (Zhang et al. 2010). Future work may include exploring whether this is also the case in the reproductive system. The importance of this is that powerful glucocorticoids like betamethasone and dexamethasone are already in clinical use and are administered to women at risk of preterm labour as they have been demonstrated to have a highly beneficial effect in reducing the incidence of cerebroventricular haemorrhage, necrotising enterocolitis, respiratory distress syndrome and neonatal death (Roberts and Dalziel 2006). However, antenatally administered steroids also been shown to have detrimental effects, having been linked to intrauterine growth restriction (Seckl 2004). A clearer picture of the functional effects of glucocorticoid-regulated ANXA1 would seem very important to help guide our use of glucocorticoids antenatally.

Discussion so far has focused on the state of pregnancy and parturition. A vitally important part of the whole process is of course resolution following delivery and a return to the physiologically different state of non-pregnancy. Immediately after the delivery of the infant comes delivery of the placenta, involution of the uterus and a gradual return of the uterus to its pre-pregnant state. It may be that pro-resolution mediators are involved in this process and that upregulation of their receptors during labour is a reflection of the body preparing itself for the next part of the process so that this may be carried out in a timely fashion to reduce complications of the post-partum period such as primary and secondary post-partum haemorrhage. Future work may include studying samples from women in the post-partum period, monitoring LXA4 and ANXA1 levels in the hours and days following delivery and using this information to guide further work as to the role of pro-resolution mediators post-natally.

7.4. CONCLUSIONS

This thesis has provided novel findings regarding the role of pro-resolution mediators in pregnancy and parturition. I hypothesised that the onset of parturition is influenced not only by pro-inflammatory mediators, but also pro-resolution and anti-inflammatory mediators. The evidence presented in this thesis appears to support this hypothesis. Circulating LXA4 protein levels were elevated in pregnancy, but fall towards term, suggesting a 'brake' on inflammation during pregnancy that is eased off as labour approaches. FPR1 and FPR2/ALX mRNA expression were increased during labour. This represents a mechanism by which ANXA1 and LXA4 may have increased effects to try and counteract the inflammatory cascade of labour. It may also be that FPR2/ALX becomes increasingly available to pro-inflammatory mediators, overwhelming the actions of LXA4 and ANXA1.

Furthermore, I hypothesised that stimulation of non-labouring reproductive human tissue by stress mediators such as lipopolysaccharide, hypoxia and cortisol would result in a rise in pro-resolution/anti-inflammatory mediators, in order to attenuate the effect of the external stimulus and maintain the quiescent state of pregnancy. The pro-inflammatory stimulants LPS and hypoxia did not result in an increase of secreted LXA4 and mRNA expression of ALOX5 was attenuated by these stimuli. No statistically significant effect was shown on FPR2/ALX and further work will help to clarify the interactions between pro-inflammatory mediators and LXA4 and ANXA1. Cortisol did not affect mRNA expression of ANXA1, but did upregulate mRNA expression of FPR1 and FPR2/ALX, which could represent a mechanism by which ANXA1 could have increased action, thus supporting this hypothesis.

The evidence presented suggests that the resolution of inflammation is important in pregnancy and thus future work will aim to examine this role further to determine what beneficial effects may be brought from it. Ultimately it would seem that solutions for preterm and abnormal term labour will not focus on a mono-therapy, such as an agent purely to halt preterm activity, but will comprise multi-faceted approaches aiming to target the abnormal process from various angles. I hope and believe that in the future we will be able to utilise the knowledge being gained from

the study of pro-resolution mediators to devise novel strategies to treat preterm birth and abnormal labour at term, and thus reduce the tremendous burden of these disorders on women and their infants.

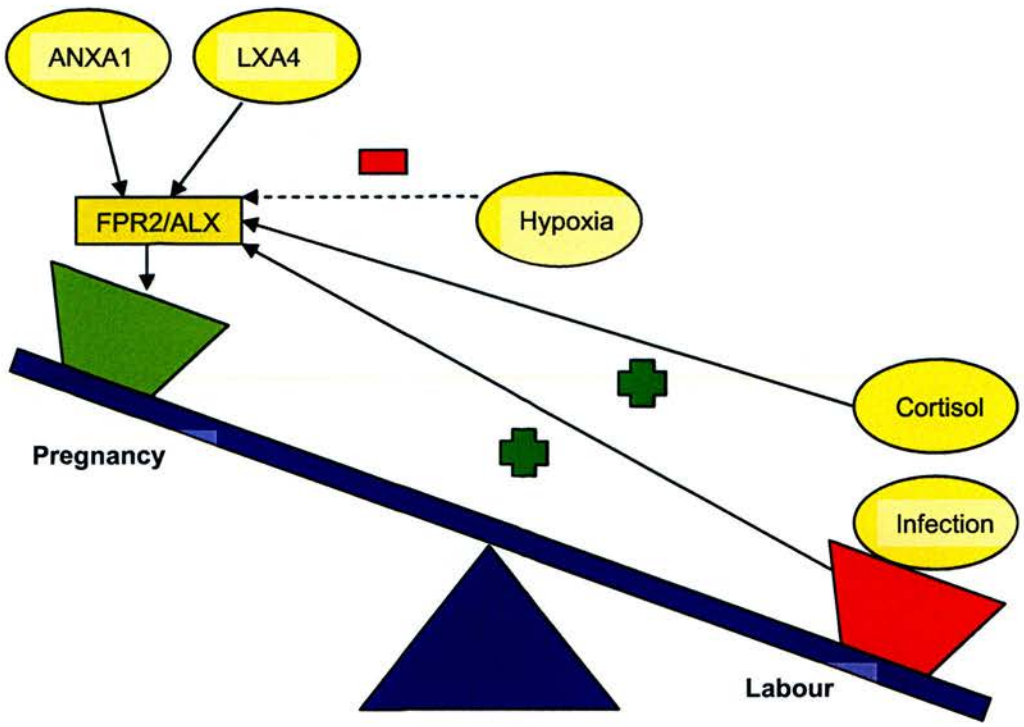


Figure 7.1

Diagrammatic Representation of Conclusions

This figure represents the 'balance' between pregnancy and labour tipping towards labour. LXA4 levels increase during pregnancy, falling towards the end of gestation, suggesting a pro-pregnancy effect. Labour and cortisol result in increased expression of FPR2/ALX. This represents a mechanism by which ANXA1 and LXA4 may have increased effects to try and counteract the inflammatory cascade of labour. It may also be that FPR2/ALX becomes increasingly available to pro-inflammatory mediators, overwhelming the actions of LXA4 and ANXA1. The dashed line between hypoxia and FPR2/ALX represents that the findings were not statistically significant but that the expression trended towards a decrease in FPR2/ALX expression with hypoxia.

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APPENDIX 1: MATERIALS

Sources for all reagents and kits used in the experiments described in this thesis are listed below.

TISSUE COLLECTION	SOURCE
RPMI 1640 Culture medium	Sigma-Aldrich, Poole, UK
10% Neutral Buffered Formalin	See recipe Appendix 3
37% Formaldehyde Solution	Sigma-Aldrich, Poole, UK
Phosphate Buffered Saline (PBS)	Sigma-Aldrich, Poole, UK
RNAlater solution	Qiagen, Crawley, W Sussex, UK
70% IMS	See recipe Appendix 2
Starstedt Monvette blood tubes	Starstedt AG & Co, Numbrecht, Germany

TISSUE CULTURE	SOURCE
RPMI 1640 Culture medium	Sigma-Aldrich, Poole, UK
Penicillin/Streptomycin	Invitrogen, Paisley, UK
Phosphate Buffered Saline (PBS)	Invitrogen, Paisley, UK
24 well culture plates	Corning, NY, USA
Lipopolysaccharide, from Salmonella abortus equii	ALEXIS Biochemicals, AXXORA, San Diego, CA
Lipoxin A4	Calbiochem, La Jolla, CA, USA

IMMUNOHISTOCHEMISTRY, GENERAL	SOURCE
Absolute ethanol	VWR International, Leuven, Belgium
Xylene	Fisher Scientific, Loughborough, UK
Citrate buffer	See recipe Appendix 2
Citric acid (monohydrate)	Sigma-Aldrich, Poole, UK
Hydrogen peroxide	VWR International, Leuven, Belgium
Methanol	Fisher Scientific, Loughborough, UK
Tris Buffered Saline (TBS)	See recipe Appendix 2
Tris(hydroxymethyl)methylamine	VWR International, Belgium
Normal goat serum	Sigma-Aldrich, Poole, UK
Phosphate Buffered Saline (PBS)	See recipe Appendix 2
Bovine Serum Albumin	Sigma-Aldrich, Poole, UK
NGS/PBS/BSA blocking serum	See recipe Appendix 2
Avidin-Biotin block	Vector Labs Inc, Burlinghame, CA, USA
Streptavadin-HRP	Boehringer Ingelheim, Berks, UK
ImmPACT DAB substrate	Vector Labs Inc, Burlinghame, CA, USA
Haematoxylin	Sigma-Aldrich, Poole, UK
Scott's tap water	See recipe Appendix 2
Sodium bicarbonate	Sigma-Aldrich, Poole, UK
Magnesium sulphate	Sigma-Aldrich, Poole, UK
Tyramide Signal Amplification Kits: TSA™ –Plus Cyanine 3 System (Red) TSA™ –Plus Fluorescien System (Green)	Perkin Elmer Inc, Waltham, MA, USA

DAPI	Sigma-Aldrich, Poole, UK
Microscopy slides SuperFrost® Plus	VWR International, Leuven, Belgium
Coverslips	VWR International, Leuven, Belgium
Permafluor	Thermoscientific, Rockford, IL, USA
Pertex	CellPath Ltd, Powys, UK

IMMUNOHISTOCHEMISTRY, ANTIBODIES	**SOURCE**
Rabbit anti-FPR2/ALX	Lifespan Biosciences, Seattle, USA
Goat anti-rabbit biotinylated	Abcam, Cambridge, UK
Rabbit monoclonal IgG	Abcam, Cambridge, UK
Mouse anti-AnnexinA1	BD Biosciences, Oxford, UK
Goat anti-mouse biotinylated	Abcam, Cambridge, UK
Mouse monoclonal IgG	Abcam, Cambridge, UK
Mouse anti-neutrophil elastase	Dako, Glostrup, Denmark
Goat anti-mouse peroxidase + Fab	Abcam, Cambridge, UK
Goat anti-rabbit peroxidase + Fab	Abcam, Cambridge, UK

QUANTITATIVE RT-PCR	**SOURCE**
Trizol	Sigma-Aldrich, Poole, UK
RNeasy columns	Qiagen, Crawley, W Sussex, UK
Chloroform	Sigma-Aldrich, St Louis, MO, USA
Ethanol	VWR International, Leuven, Belgium
DNase digestion kit	Qiagen, Crawley, W Sussex, UK
DNase free RNase free water	Sigma-Aldrich, Irvine, UK
GeneAmp RNA PCR kit	Applied Biosystems, Warrington, Cheshire, UK
RT-PCR Mastermix, TaqMan Fast Universal Mastermix	Applied Biosystems, Warrington, Cheshire, UK
RT-PCR Mastermix used in ALOX12 and ALOX15 experiment, TaqMan Fast Universal PCR Mastermix x2	Invitrogen, Paisley, UK
18S primers and probes mix	Applied Biosystems, Warrington, Cheshire, UK
TaqMan Fast optical PCR plates	Applied Biosystems, Warrington, Cheshire, UK
Adhesive optical films	Applied Biosystems, Warrington, Cheshire, UK
FPR2/ALX primers	Eurogentec, Southampton, UK
ALOX5 primers	Eurogentec, Southampton, UK
ALOX12 primers	Eurogentec, Southampton, UK
ALOX15 primers	Eurogentec, Southampton, UK
Universal Probe Library Probes	Roche, Indianapolis, IN, USA
E-Selectin primers and probes	Applied Biosystems, Warrington, Cheshire, UK
CCL2 primers and probes	Applied Biosystems, Warrington,

	Cheshire, UK
CSF3 primers and probes	Applied Biosystems, Warrington, Cheshire, UK
MMP1 primers and probes	Applied Biosystems, Warrington, Cheshire, UK
ICAM1 primers and probes	Applied Biosystems, Warrington, Cheshire, UK
FPR1 primers and probes	Sigma-Aldrich, Poole, UK
ANXA1 primers and probes	Sigma-Aldrich, Poole, UK
11βHSD1 primers and probes	Applied Biosystems, Warrington, Cheshire, UK
11BHSD2 primers and probes	Applied Biosystems, Warrington, Cheshire, UK
VEGF	Eurogentec, Southampton, UK

LXA4 ELISA	**SOURCE**
ELISA kit	Neogen Corporation, Lexington, KY, USA

APPENDIX 2: RECIPES FOR SOLUTIONS

All dilutions are in deionized water, unless otherwise stated.

0.1 M Citrate buffer

42.02g Citric Acid (monohydrate)

1900mls water

Made up to 2 litres and pH 6.0 adding concentrated NaOH as necessary.

Used at 0.01M, diluting in distilled water.

0.5M TRIS-HCl stock x 10

121.14g Tris(hydroxymethyl)methylamine

1800mls water

Made up to 2 litres and pH 7.4, adding concentrated HCl as necessary.

0.05M TBS

100mls 0.5M Tris-HCl (recipe above)

900mls water

8.5g Sodium Chloride

10 % Neutral Buffered Formalin

37% Formaldehyde Solution diluted 1 in 10 with 0.01M PBS

Phosphate buffered saline

5 PBS tablets (Sigma-Aldrich, Poole, Dorset, UK)

1000mls water

pH 7.4-7.6

NGS/PBS/BSA blocking serum

20ml Normal Goat Serum

80 ml Phosphate Buffered Saline

5g Bovine Serum Albumin

Scott's Tap Water

3.2g Sodium Bicarbonate (Sigma-Aldrich, Poole, Dorset, UK)

36.1g Magnesium Sulphate (Sigma-Aldrich, Poole, Dorset, UK)

1000mls water

APPENDIX 3: LISTS OF UPREGULATED AND DOWNREGULATED GENES, LPS vs VEHICLE

The following table lists all differentially expressed upregulated genes in the comparison of LPS vs Vehicle from the microarray data reported in Chapter 4.

Symbol	Gene name	Fold change	FDR corrected P-value
SELE	E selectin	53.07	2.85E-13
CCL3	chemokine (C-C motif) ligand 3	27.07	3.51E-13
CX3CL1	chemokine (C-X3-C motif) ligand 1	24.93	5.89E-13
CCL4L2	chemokine (C-C motif) ligand 4-like 2	21.19	1.81E-14
CCL3L1	chemokine (C-C motif) ligand 3-like 1	23.16	4.46E-12
LTB	lymphotoxin beta (TNF superfamily, member 3)	19.27	6.45E-13
CCL3L1	chemokine (C-C motif) ligand 3-like 1	19.23	4.76E-12
CCL3L3	chemokine (C-C motif) ligand 3-like 3	22.76	6.72E-11
IL1A	interleukin 1, alpha	18.35	7.21E-12
IL1B	interleukin 1, beta	42.50	5.96E-10
CCL20	chemokine (C-C motif) ligand 20	23.13	5.38E-10
TRAF1	TNF receptor-associated factor 1	7.79	1.18E-12
CSF3	colony stimulating factor 3 (granulocyte)	13.57	2.02E-10
TNFAIP2	tumor necrosis factor, alpha-induced protein 2	10.75	1.43E-10
CCL4L1	chemokine (C-C motif) ligand 4-like 1	10.01	1.07E-10
IL23A	interleukin 23, alpha subunit p19	22.73	6.81E-09
LTB	lymphotoxin beta (TNF superfamily, member 3)	6.93	8.49E-10
CCL8	chemokine (C-C motif) ligand 8	9.46	6.98E-09
GCH1	GTP cyclohydrolase 1	6.87	5.54E-09
ADORA2A	adenosine A2a receptor	5.07	3.64E-10
SLC2A6	solute carrier family 2 (facilitated glucose transporter), member 6	4.92	2.02E-10
C15orf48	chromosome 15 open reading frame 48	5.85	4.20E-09
CSF3	colony stimulating factor 3 (granulocyte)	17.63	2.51E-08
IL1RN	interleukin 1 receptor antagonist	6.78	7.98E-09
TNFRSF4	tumor necrosis factor receptor superfamily, member 4	4.95	8.38E-10
CCL3L1	chemokine (C-C motif) ligand 3-like 1	9.73	2.62E-08
CXCL2	chemokine (C-X-C motif) ligand 2	10.12	4.59E-08
TNIP1	TNFAIP3 interacting protein 1	3.94	2.30E-11
UBD	ubiquitin D	12.39	5.35E-08
CFB	complement factor B	6.38	2.24E-08
LIPG	lipase, endothelial	6.35	2.44E-08
LIPG	lipase, endothelial	4.44	7.06E-09
IL7R	interleukin 7 receptor	5.26	3.23E-08

Symbol	Gene name	Fold change	FDR corrected P-value
CSF2	colony stimulating factor 2 (granulocyte-macrophage)	9.58	1.50E-07
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	3.42	2.94E-11
VCAM1	vascular cell adhesion molecule 1	8.90	1.39E-07
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	3.18	2.75E-13
G0S2	G0/G1switch 2	6.41	6.50E-08
CITED4	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4	4.78	4.58E-08
GPR84	G protein-coupled receptor 84	4.49	4.58E-08
IL32	interleukin 32	3.70	5.28E-09
IRAK2	interleukin-1 receptor-associated kinase 2	3.40	2.73E-09
ZC3H12A	zinc finger CCCH-type containing 12A	4.09	3.64E-08
HCK	hemopoietic cell kinase	3.27	8.49E-10
NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	3.66	9.23E-09
MEOX1	mesenchyme homeobox 1	3.73	1.85E-08
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	6.90	4.37E-07
C15orf48	chromosome 15 open reading frame 48	3.77	4.91E-08
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	7.57	6.78E-07
TNFAIP6	tumor necrosis factor, alpha-induced protein 6	4.11	1.82E-07
CXCL10	chemokine (C-X-C motif) ligand 10	17.36	1.02E-06
RIPK2	receptor (TNFRSF)-interacting serine-threonine kinase 1	3.66	4.91E-08
NINJ1	Ninjurin 1	3.00	4.29E-09
EBI3	Epstein-Barr induced gene 3	3.02	7.98E-09
LINCR	neuralized homolog 3 (Drosophila) pseudogene	5.14	7.23E-07
IL7R	Interleukin 7 receptor	3.87	2.87E-07
PRIC285	peroxisomal proliferator-activated receptor A interacting complex 285	4.51	5.83E-07
IL18R1	Interleukin 18 receptor 1	2.90	1.94E-08
TIFA	TRAF-interacting protein with forkhead-associated domain	2.65	2.23E-09
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	3.32	2.09E-07
SLCO5A1	solute carrier organic anion transporter family, member 5A1	2.72	7.98E-09
ALCAM	activated leukocyte cell adhesion molecule	2.93	4.91E-08

Symbol	Gene name	Fold change	FDR corrected P-value
TNIP3	TNFAIP3 interacting protein 3	5.18	1.34E-06
APOL3	Apolipoprotein L, 3	3.13	2.31E-07
CD40	CD40 molecule, TNF receptor superfamily member 5	3.23	3.13E-07
KYNU	kynureninase	2.84	1.06E-07
IL15RA	interleukin 15 receptor, alpha	2.82	9.30E-08
BIRC3	baculoviral IAP repeat containing 3	3.56	8.06E-07
IFNGR2	interferon gamma receptor 2 (interferon gamma transducer 1)	2.60	1.98E-08
PIK3C2B	phosphoinositide-3-kinase, class 2, beta polypeptide	2.50	2.26E-09
IFNAR2	interferon (alpha, beta and omega) receptor 2	2.57	8.02E-09
RND1	Rho family GTPase 1	3.83	1.17E-06
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	5.04	1.91E-06
SAMD9L	sterile alpha motif domain containing 9-like	3.04	3.80E-07
FRMD4A	FERM domain containing 4A	2.69	9.80E-08
IFIH1	interferon induced with helicase C domain 1	3.80	1.17E-06
PARP14	poly (ADP-ribose) polymerase family, member 14	2.97	3.63E-07
RELB	v-rel reticuloendotheliosis viral oncogene homolog B	2.40	8.25E-10
CCL5	chemokine (C-C motif) ligand 5	6.63	2.71E-06
NOD2	nucleotide-binding oligomerization domain containing 2	4.00	1.53E-06
NLF2	C2 calcium-dependent domain containing 4B	4.56	2.12E-06
RHBDF2	rhomboid 5 homolog 2 (Drosophila)	2.32	2.30E-10
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	5.67	2.71E-06
KYNU	kynureninase	2.71	2.29E-07
GBP1	guanylate binding protein 1, interferon-inducible	4.36	2.17E-06
TMEM171	transmembrane protein 171	3.30	1.17E-06
SLC7A2	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	4.59	2.39E-06
FAS	(TNF receptor superfamily, member 6)	2.61	1.82E-07
CCL5	chemokine (C-C motif) ligand 5	6.53	3.61E-06
CD83	chemokine (C-C motif) ligand 5	3.25	1.22E-06
PDE4B	phosphodiesterase 4B, cAMP-specific	2.61	2.31E-07
LOC653778	similar to solute carrier family 25, member 37	2.66	3.37E-07
NFS1	nitrogen fixation 1 homolog (S. cerevisiae)	4.58	3.01E-06
EHD1	EH-domain containing 1	2.47	4.99E-08
TAP1	transporter 1,ATP-binding cassette,subfam B	2.58	2.29E-07

Symbol	Gene name	Fold change	FDR corrected P-value
GCH1	GTP cyclohydrolase 1	2.84	1.02E-06
CXCR7	chemokine (C-X-C motif) receptor 7	2.68	6.61E-07
IQCA1	IQ motif containing with AAA domain 1	2.81	1.10E-06
SLAMF1	signaling lymphocytic activation molecule family member 1	5.32	6.22E-06
BDKRB2	Bradykinin receptor B2	3.09	1.57E-06
MEOX1	mesenchyme homeobox 1	2.55	3.86E-07
MCOLN2	Mucolipin 2	2.60	9.85E-07
LRRC49	leucine rich repeat containing 49	2.18	4.20E-09
RHBDF2	rhomboid 5 homolog 2 (Drosophila)	2.21	1.98E-08
ICAM1	intercellular adhesion molecule 1	2.59	1.02E-06
ISG20	interferon stimulated exonuclease gene 20kDa	2.99	2.18E-06
HLA-F	major histocompatibility complex, class I, F	2.33	1.99E-07
ICOSLG	inducible T-cell co-stimulator ligand	2.20	2.28E-08
CYP27B1	cytochrome P450, family 27, subfamily B, polypeptide 1	3.74	4.41E-06
CD40	CD40 molecule	2.31	1.82E-07
CCL2	chemokine (C-C motif) ligand 2	3.49	3.77E-06
CD83	CD83 molecule	3.12	2.91E-06
BDKRB1	bradykinin receptor B1	4.16	7.50E-06
VCAM1	vascular cell adhesion molecule 1	4.07	7.22E-06
PDGFB	platelet-derived growth factor beta polypeptide	2.95	3.21E-06
LOC654103	similar to solute carrier family 25, member 37	2.53	1.39E-06
LRFN5	leucine rich repeat and fibronectin type III domain containing 5	2.28	5.10E-07
TNF	Tumor necrosis factor	4.03	8.24E-06
MOBKL2C	MOB kinase activator 3C	2.12	4.59E-08
SLC25A37	solute carrier family 25, member 37	2.77	3.11E-06
DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58	2.97	4.46E-06
S100A9	S100 calcium binding protein A9	2.49	1.55E-06
C1QTNF1	C1q and tumor necrosis factor related protein 1	2.40	1.37E-06
GBP4	guanylate binding protein 4	3.95	1.05E-05
MMP1	Matrix metalloproteinase 1	2.45	1.41E-06
IL4I1	interleukin 4 induced 1	2.13	1.85E-07
STX11	syntaxin 11	2.44	1.55E-06
GBP1	guanylate binding protein 1, interferon-inducible	4.32	1.21E-05
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	2.39	1.55E-06

Symbol	Gene name	Fold change	FDR corrected P-value
SRGN	serglycin	2.51	2.47E-06
LILRB2	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 2	2.58	3.31E-06
SOCS1	suppressor of cytokine signaling 1	2.59	3.39E-06
NBN	nibrin	2.48	2.39E-06
CXCL5	chemokine (C-X-C motif) ligand 5	4.99	1.66E-05
TAP2	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	2.26	1.41E-06
PI3	peptidase inhibitor 3, skin-derived	3.86	1.43E-05
CXCL5	chemokine (C-X-C motif) ligand 5	5.63	2.13E-05
CBR3	carbonyl reductase 3	2.96	7.85E-06
LIF	leukemia inhibitory factor (cholinergic differentiation factor)	3.73	1.33E-05
A4GALT	alpha 1,4-galactosyltransferase	2.02	1.06E-07
CYLD	cylindromatosis (turban tumor syndrome)	2.13	7.26E-07
IQCA1	IQ motif containing with AAA domain 1	2.87	8.23E-06
BIRC3	baculoviral IAP repeat containing 3	2.15	1.01E-06
STAT5A	signal transducer and activator of transcription 5A	2.25	1.72E-06
PSTPIP2	proline-serine-threonine phosphatase interacting protein 2	2.29	2.21E-06
TRIM21	tripartite motif containing 21	2.12	9.29E-07
STAT4	signal transducer and activator of transcription 4	2.55	5.24E-06
DBI	diazepam binding inhibitor (GABA receptor modulator, acyl-CoA binding protein)	2.29	2.31E-06
SLCO3A1	solute carrier organic anion transporter family, member 3A1	2.03	3.19E-07
TAPBP	TAP binding protein (tapasin)	2.09	6.79E-07
XAF1	XIAP associated factor 1	3.06	1.24E-05
KCNN2	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	3.15	1.37E-05
AQP9	aquaporin 9	2.68	9.66E-06
LOC647650	hypothetical protein LOC647650	2.58	7.72E-06
PLAU	plasminogen activator, urokinase	2.56	7.84E-06
SLC8A3	solute carrier family 8 (sodium/calcium exchanger), member 3	4.23	2.80E-05
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor,	2.16	2.91E-06
SPRY4	sprouty homolog 4 (Drosophila)	2.12	2.81E-06
CD200	CD200 molecule	2.10	2.36E-06

Symbol	Gene name	Fold change	FDR corrected P-value
S1PR3	sphingosine-1-phosphate receptor 3	2.02	1.41E-06
CD48	CD48 molecule	2.18	4.94E-06
GJB2	gap junction protein, beta 2, 26kDa	3.18	3.18E-05
IL32	Interleukin 32	2.69	2.19E-05
IRF1	interferon regulatory factor 1	2.98	3.17E-05
SLC41A1	solute carrier family 41, member 1	2.28	1.08E-05
LOC730249	immunoresponsive 1 homolog	7.19	7.23E-05
PDGFB	platelet-derived growth factor beta polypeptide	2.36	1.41E-05
DKFZp686O24166	B7 homolog 6	2.11	6.64E-06
PID1	phosphotyrosine interaction domain containing 1	2.11	6.94E-06
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	4.55	6.69E-05
CXCR7	chemokine (C-X-C motif) receptor 7	2.22	1.19E-05
MSC	musculin	2.83	4.04E-05
TMEM194	transmembrane protein 194A	2.20	1.31E-05
SRGN	serglycin	2.60	3.19E-05
TNFAIP8	tumor necrosis factor, alpha-induced protein 8	3.72	6.39E-05
CA8	carbonic anhydrase VIII	2.23	1.57E-05
AMPD3	adenosine monophosphate deaminase 3	2.15	1.46E-05
IL8	Interleukin 8	3.86	8.58E-05
RGS16	regulator of G-protein signaling 16	2.59	4.19E-05
GJD3	gap junction protein, delta 3, 31.9kDa	2.21	2.22E-05
NLRP3	NLR family, pyrin domain containing 3	2.39	3.26E-05
CXCL6	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	3.84	0.0001
TREM1	triggering receptor expressed on myeloid cells 1	2.16	2.38E-05
ABTB2	ankyrin repeat and BTB (POZ) domain containing 2	2.18	2.68E-05
XAF1	XIAP associated factor 1	2.36	3.73E-05
JAG1	Jagged 1	2.32	3.62E-05
S100A3	S100 calcium binding protein A3	2.11	1.99E-05
OASL	2'-5'-oligoadenylate synthetase-like	4.02	0.000124
RTP4	receptor (chemosensory) transporter protein	2.26	3.53E-05
FGF2	fibroblast growth factor 2 (basic)	2.01	1.43E-05
NKX3-1	NK3 homeobox 1	2.43	5.99E-05
CLIC6	chloride intracellular channel 6	2.11	2.75E-05
CXCL6	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	5.47	0.000196

Symbol	Gene name	Fold change	FDR corrected P-value
CYB5A	cytochrome b5 type A (microsomal)	2.02	1.99E-05
TNFRSF6B	tumor necrosis factor receptor superfamily, member 6b, decoy	2.30	5.60E-05
SGPP2	sphingosine-1-phosphate phosphatase 2	3.46	0.00014
NLRP3	NLR family, pyrin domain containing 3	2.42	6.59E-05
SAMSN1	SAM domain, SH3 domain and nuclear localization signals 1	2.49	7.78E-05
IL6	Interleukin 6	4.12	0.000216
OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa	3.19	0.000261
HERC6	hect domain and RLD 6	3.15	0.000305
NAMPT	nicotinamide phosphoribosyltransferase	2.05	9.66E-05
EPSTI1	epithelial stromal interaction 1 (breast)	3.11	0.000308
SOX7	SRY (sex determining region Y)-box 7	2.02	8.67E-05
NUAK2	NUAK family, SNF1-like kinase, 2	2.05	0.000104
SLC39A8	solute carrier family 39 (zinc transporter), member 8	2.59	0.000259
F3	coagulation factor III (thromboplastin, tissue factor)	2.09	0.000168
LOC728715	ovostatin homolog 2-like	2.32	0.000249
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	3.31	0.000511
TMEM166	transmembrane protein 166	2.09	0.000195
CD82	CD82 molecule	2.08	0.000207
FSTL3	follistatin-like 3 (secreted glycoprotein)	2.17	0.000286
WTAP	Wilms tumor 1 associated protein	2.06	0.000278
MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78	2.76	0.000632
NGF	nerve growth factor (beta polypeptide)	2.17	0.000442
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	2.57	0.000658
CD38	CD38 molecule	2.28	0.000565
FAS	(TNF receptor superfamily, member 6)	2.02	0.000381
IRF7	interferon regulatory factor 7	2.19	0.00054
RASSF5	Ras association (RalGDS/AF-6) domain family member 5	2.44	0.00079
CCL19	chemokine (C-C motif) ligand 19	2.57	0.000855
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	2.74	0.000978
SSTR2	somatostatin receptor 2	2.06	0.000518
IFI44	interferon-induced protein 44	2.53	0.000863

The following table lists all differentially expressed downregulated genes in the comparison of LPS vs Vehicle from the microarray data reported in Chapter 4.

Symbol	Gene name	Fold change	FDR corrected P-value
GRRP1	glycine/arginine rich protein 1	-5.72	9.87E-09
PPP1R16B	protein phosphatase 1, regulatory (inhibitor) subunit 16B	-2.79	5.38E-10
INHBB	inhibin, beta B	-3.34	1.38E-07
HAVCR2	hepatitis A virus cellular receptor 2	-2.88	6.02E-08
RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	-2.48	5.85E-11
LHX6	LIM homeobox 6	-2.72	1.92E-07
CARD10	caspase recruitment domain family, member 10	-2.56	3.64E-08
RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	-2.32	7.98E-09
GCHFR	GTP cyclohydrolase I feedback regulator	-2.50	2.55E-07
FPR3	formyl peptide receptor 3	-3.09	2.14E-06
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble	-2.68	1.53E-06
IFI30	interferon, gamma-inducible protein 30	-2.58	1.17E-06
MTUS1	microtubule associated tumor suppressor 1	-2.47	9.92E-07
GAS1	growth arrest-specific 1	-2.55	1.23E-06
GIMAP6	GTPase, IMAP family member 6	-2.28	1.10E-06
ICAM2	intercellular adhesion molecule 2	-2.46	2.12E-06
MFNG	MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	-2.16	9.29E-07
FUCA1	fucosidase, alpha-L- 1, tissue	-2.67	6.45E-06
C6orf141	chromosome 6 open reading frame 141	-2.26	1.85E-06
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1	-2.71	7.72E-06
KBTBD11	kelch repeat and BTB (POZ) domain containing 11	-2.88	1.09E-05
NOSTRIN	nitric oxide synthase trafficker	-2.03	4.89E-07
MAN1C1	mannosidase, alpha, class 1C, member 1	-2.10	1.34E-06
CPLX1	complexin 1	-2.22	3.26E-06
HSPA12B	heat shock 70kD protein 12B	-2.01	1.10E-06
MALL	mal, T-cell differentiation protein-like	-2.12	3.37E-06
SDS	serine dehydratase	-3.30	3.31E-05
GALM	galactose mutarotase (aldose 1-epimerase)	-2.05	2.75E-06
RNASE6	ribonuclease, RNase A family, k6	-2.60	2.07E-05
NPL	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)	-2.25	9.83E-06
PDE7B	phosphodiesterase 7B	-2.04	3.27E-06
ADAP2	ArfGAP with dual PH domains 2	-2.01	3.31E-06

Symbol	Gene name	Fold change	FDR corrected P-value
MBP	myelin basic protein	-2.16	1.21E-05
DACH1	dachshund homolog 1 (Drosophila)	-2.10	1.03E-05
NPL	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)	-2.15	1.59E-05
CD34	CD34 molecule	-2.23	2.62E-05
MTUS1	microtubule associated tumor suppressor 1	-2.09	1.41E-05
MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A	-2.05	1.24E-05
TEK	TEK tyrosine kinase, endothelial	-2.18	2.04E-05
CD34	CD34 molecule	-2.03	1.24E-05
CYTL1	cytokine-like 1	-2.48	4.07E-05
TPD52L1	tumor protein D52-like 1	-2.03	1.44E-05
CYYR1	cysteine/tyrosine-rich 1	-2.15	2.67E-05
LAPTM5	lysosomal protein transmembrane 5	-2.04	1.78E-05
PROX1	prospero homeobox 1	-2.20	3.29E-05
STC2	stanniocalcin 2	-2.05	2.79E-05
VAMP8	vesicle-associated membrane protein 8 (endobrevin)	-2.01	2.79E-05
HLA-DMB	major histocompatibility complex, class II, DM beta	-2.19	5.62E-05
CD300A	CD300a molecule	-2.09	4.07E-05
GRAP	GRB2-related adaptor protein 2	-2.03	3.34E-05
GRRP1	glycine/arginine rich protein 1	-5.72	9.87E-09
PPP1R16B	protein phosphatase 1, regulatory (inhibitor) subunit 16B	-2.79	5.38E-10
INHBB	inhibin, beta B	-3.34	1.38E-07
HAVCR2	hepatitis A virus cellular receptor 2	-2.88	6.02E-08
RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	-2.48	5.85E-11
LHX6	LIM homeobox 6	-2.72	1.92E-07
CARD10	caspase recruitment domain family, member 10	-2.56	3.64E-08
RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	-2.32	7.98E-09
GCHFR	GTP cyclohydrolase I feedback regulator	-2.50	2.55E-07
FPR3	formyl peptide receptor 3	-3.09	2.14E-06
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble	-2.68	1.53E-06
IFI30	interferon, gamma-inducible protein 30	-2.58	1.17E-06
MTUS1	microtubule associated tumor suppressor 1	-2.47	9.92E-07
GAS1	growth arrest-specific 1	-2.55	1.23E-06
GIMAP6	GTPase, IMAP family member 6	-2.28	1.10E-06
ICAM2	intercellular adhesion molecule 2	-2.46	2.12E-06
MFNG	MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	-2.16	9.29E-07
FUCA1	fucosidase, alpha-L- 1, tissue	-2.67	6.45E-06
C6orf141	chromosome 6 open reading frame 141	-2.26	1.85E-06

Symbol	Gene name	Fold change	FDR corrected P-value
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1	-2.71	7.72E-06
KBTBD11	kelch repeat and BTB (POZ) domain containing 11	-2.88	1.09E-05
NOSTRIN	nitric oxide synthase trafficker	-2.03	4.89E-07
MAN1C1	mannosidase, alpha, class 1C, member 1	-2.10	1.34E-06
CPLX1	complexin 1	-2.22	3.26E-06
HSPA12B	heat shock 70kD protein 12B	-2.01	1.10E-06
MALL	mal, T-cell differentiation protein-like	-2.12	3.37E-06
SDS	serine dehydratase	-3.30	3.31E-05
GALM	galactose mutarotase (aldose 1-epimerase)	-2.05	2.75E-06
RNASE6	ribonuclease, RNase A family, k6	-2.60	2.07E-05
NPL	N-acetylneuraminate pyruvate lyase	-2.25	9.83E-06
PDE7B	phosphodiesterase 7B	-2.04	3.27E-06
ADAP2	ArfGAP with dual PH domains 2	-2.01	3.31E-06
MBP	myelin basic protein	-2.16	1.21E-05
DACH1	dachshund homolog 1 (Drosophila)	-2.10	1.03E-05
NPL	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)	-2.15	1.59E-05
CD34	CD34 molecule	-2.23	2.62E-05
MTUS1	microtubule associated tumor suppressor 1	-2.09	1.41E-05
MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A	-2.05	1.24E-05
TEK	TEK tyrosine kinase, endothelial	-2.18	2.04E-05
CD34	CD34 molecule	-2.03	1.24E-05
CYTL1	cytokine-like 1	-2.48	4.07E-05
TPD52L1	tumor protein D52-like 1	-2.03	1.44E-05
CYYR1	cysteine/tyrosine-rich 1	-2.15	2.67E-05
LAPTM5	lysosomal protein transmembrane 5	-2.04	1.78E-05
PROX1	prospero homeobox 1	-2.20	3.29E-05
STC2	stanniocalcin 2	-2.05	2.79E-05
VAMP8	vesicle-associated membrane protein 8 (endobrevin)	-2.01	2.79E-05
HLA-DMB	major histocompatibility complex, class II, DM beta	-2.19	5.62E-05
CD300A	CD300a molecule	-2.09	4.07E-05
GRAP	GRB2-related adaptor protein 2	-2.03	3.34E-05
PTP4A3	protein tyrosine phosphatase type IVA, member 3	-2.02	4.38E-05
FAM124B	family with sequence similarity 124B	-2.36	0.000101
CLEC14A	C-type lectin domain family 14, member A	-2.16	9.47E-05
GIMAP7	GTPase, IMAP family member 7	-2.15	0.0001
SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	-2.97	0.000262

Symbol	Gene name	Fold change	FDR corrected P-value
ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	-2.03	0.000129
MGST2	microsomal glutathione S-transferase 2	-2.02	0.000148
GDF15	growth differentiation factor 15	-2.1	0.000237
DHRS9	dehydrogenase/reductase (SDR family) member 9	-2.64	0.000756
SEPP1	selenoprotein P, plasma, 1	-2.06	0.00041

APPENDIX 4: PUBLICATIONS, PRESENTATIONS AND POSTERS RELATING TO THIS THESIS

PUBLICATIONS

Golightly, E., Jabbour, H. J., Norman, J. E. (2011). "Endocrine immune interactions in human parturition." Mol Cell Endocrinol **335** (1): 52-9

Maldonado-Perez, D.. Golightly, E., Denison, F. C., Jabbour, H. J., Norman, J. E. (2010). "A role for lipoxin A4 as anti-inflammatory and proresolution mediator in human parturition." Faseb J. **25** (2): 569-75

ORAL PRESENTATIONS

Expression of Annexin A1 and the receptor FPR2 in labouring and non-labouring human pregnant reproductive tissues.
Presented at the Edinburgh Perinatal Festival, Edinburgh, May 2010

POSTERS

"Expression and regulation of novel anti-inflammatory agents in human reproductive tissues in labour", Golightly, E., Jabbour, H. J., Norman, J. E.
Presented at the Blair Bell Society Annual Academic Meeting, London, November 2010.

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